8-24-2018

The Effects of Poor Maternal Nutrition on Offspring Muscle Metabolism

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B.S., University of Connecticut, 2016

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Science

At the

University of Connecticut

2018

APPROVAL PAGE

Masters of Science Thesis

The Effects of Poor Maternal Nutrition on Offspring Muscle Metabolism

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2018

ACKNOWLEDGEMENTS

I would first like to acknowledge and sincerely thank my major advisor Dr. Sarah Reed. Dr. Reed has been a true mentor since I joined her lab as an undergraduate. Working under Dr. Reed has made me into a better scientist and student. I am very fortunate to have worked on a variety of projects during my four years in Reed Lab, and the lessons I have learned are invaluable. I would also like to thank my committee members Dr. Steven Zinn, Dr. Kristen Govoni, and Dr. Ji-Young Lee for their continued support and guidance throughout this process.

I would also like to thank my family and friends who were always invested in my education. My mother is one of the main reasons that I decided to continue my education, and she was always there for support while I pursued my undergraduate and then graduate degree. I am also thankful for all the friends I have made in the Animal Science department. The other graduate students as well as others in the department have made Animal Science feel like a home. I extend special thanks to Mary Wynn, Amanda Jones, Maria Hoffman, Arielle Halpern, Sambhu Pillai, Amanda Liefeld, and Stephanie Barnes. I would also like to acknowledge all the undergraduates of Reed Lab whose hard work has helped me along the way. Finally, I want to acknowledge and thank all of the faculty and staff within the Animal Science department.

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ABSTRACT

The Effects of Poor Maternal Nutrition on Offspring Muscle Metabolism
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Poor maternal nutrition during gestation alters offspring muscle composition, fiber number, postnatal growth, and stem cell function. Additionally, poor maternal nutrition impacts offspring whole body and stem cell metabolism. We hypothesized that over- or restricted-feeding during gestation would alter muscle myoblast metabolism as well as offspring Longissimus dorsi muscle (LD) metabolome. Pregnant Western White-faced ewes (n = 47) were individually fed 60% (RES), 100% (CON), or 140% (OVER) of NRC requirements starting at d 30.2 ± 0.2 of gestation. At d 90 and 135 of gestation, ewes were euthanized for fetal muscle collection. Another group of ewes were allowed to lamb and offspring were necropsied within 24 h of birth for offspring serum and muscle sample collection. C2C12 myoblasts were cultured in fetal serum to determine effects of poor maternal nutrition on cell proliferation, differentiation, and metabolism. Glycolytic function and mitochondrial respiration of C2C12 cells cultured in serum from CON, RES, and OVER lambs at birth were analyzed using the Seahorse Bioscience XF analyzer. Cell culture data were analyzed as a completely randomized design using PROC MIXED in SAS with main effects and interaction of gender and maternal diet. Mass spectrophotometry of LD samples (n = 8 fetuses per treatment per time point) identified 612 metabolites. Metabolome data were analyzed by ANOVA for main effects of treatment, time point, and their interaction. There was no observed effect ($P \leq 0.05$) of maternal diet on C2C12 proliferation, fusion index, or glycolytic function. Proton leak was increased in C2C12 cells cultured in RES serum compared with CON ($P = 0.04$). For the metabolome analysis, compared with CON, maternal over-feeding altered metabolites in 63 pathways and maternal restricted-

feeding altered metabolites in 56 pathways ($P \leq 0.05$) in the offspring. Both maternal restricted- and over-feeding altered offspring metabolites in each of the 8 major metabolic pathways including amino acid, peptide, carbohydrate, energy, lipid, nucleotide, cofactors and vitamins, and xenobiotics. Maternal over-feeding decreased concentrations of 1 phosphatidylcholine (PC) metabolite at d 90, 2 PC metabolites at d 135, and 4 PC metabolites at birth compared with CON ($P \leq 0.05$). Additionally, at d 135, maternal over-feeding increased 1 phosphatidylethanolamine (PE) metabolite while reducing 10 PE metabolites at birth compared with CON ($P \leq 0.05$). Offspring from over-fed ewes had decreased concentrations of 12 lysolipids at birth compared with CON ($P \leq 0.05$). Maternal restricted-feeding increased expression of reduced glutathione 3.07-fold at d 90 ($P = 0.008$), whereas at d 135, oxidized glutathione was decreased 21% compared with CON ($P = 0.03$). Overall, poor maternal nutrition during gestation alters offspring metabolism. Specifically, maternal over-feeding may promote increased lipid oxidation in offspring muscle, which may predispose offspring to altered lipid utilization and storage postnatally. Maternal restricted-feeding resulted in alterations in glutathione metabolism, potentially indicative of changes to redox status in these offspring.

REVIEW OF LITERATURE

Introduction

Despite advancements in production techniques and management, livestock commonly face instances of restricted- and over-feeding (Thomas and Kott, 1995; Everett-Hincks and Dodds, 2007: Hoffman et al, 2017). Restricted nutrition can often arise due to different management practices as well as variations in feed quality (Thomas and Kott, 1995; Everett-Hincks and Dodds, 2007). The quality of feed can be impacted by seasonal weather changes and/or limited forage availability or intake (Thomas and Kott, 1995; Hoffman et al., 2017). Production of sheep on a forage-based system is common, subjecting the animals to climate and forage fluctuations. Ewes are short-day breeders and typically gestate during the winter months when forage quality and abundance on the range is limited (Wu et al., 2004). Therefore, ewes raised on the range are susceptible to reduced forage quality and intake and often receive below 50% of NRC nutritional requirements during the winter months in the Western United States (Thomas and Kott, 1995). Similarly, instances of over-nutrition can result due to different management and production systems of ruminant animals. Management systems in the Northeast or at Land Grant universities may provide more than sufficient nutrients, resulting in increased feed intake and over-conditioning during the winter months (Jobgen et al., 2008). Additionally, the practice of flushing, which is increasing the amount of feed before conception to encourage superovulation in ewes (National Research Council, 1985), leads to instances of over-nutrition. Other management practices also affect sheep productivity. The practice of selecting for fecundity, or increased offspring number, has increased over the last four decades (Everett-Hincks and Dodds, 2007). This affects lamb survival since maternal nutrients and resources have to be partitioned to more offspring. Additionally, lamb mortality is greater in twin- and triplet-born lambs compared with singletons (Johnson et al., 1982; Hinch et al., 1983; Scales et al.,

1986; Hall et al., 1988). Although the increased mortality rate could be due to a variety of factors, maternal nutrition is an important contributor. Scales et al. (1986) demonstrated that when ewes bearing multiple offspring were offered additional feed during late pregnancy, lamb mortality was reduced.

Proper fetal growth and development is dependent on maternal nutritional intake during gestation. Maternal nutrition is the diet a mother consumes before pregnancy, during gestation, and continued through lactation (American Journal of Public Health, 1973). Poor maternal nutrition can negatively impact offspring growth and development and is defined as inadequate or increased nutrient availability during gestation (Hoffman et al., 2017). Both restricted- and over-feeding during gestation can result in programming of the offspring, which can lead to alterations in body size and birth weight of offspring, as well as impaired muscle growth, changes in adiposity, and altered metabolism (Hoffman et al., 2017). Additionally, poor maternal nutrition has been shown to impair overall offspring growth and health. Both epidemiological and livestock studies have demonstrated that restricted- and over-feeding have long-lasting and detrimental effects on offspring productivity and health (Hales and Barker, 2001; Wu et al., 2004). The Thrifty Phenotype Hypothesis, proposed by Hales and Barker (2001), is a classic example of programming. The hypothesis states when there is a period of maternal nutrient restriction during pregnancy, the developing fetus is exposed to inadequate nutrition, which results in programming so that the fetus is prepared to survive in an environment with limited nutrient supply later in life (Hales and Barker, 2001).

Muscle is a tissue that is susceptible to programming in response to poor maternal nutrition (Reed et al., 2014). Muscle growth is a tightly regulated process controlled by numerous intrinsic and extrinsic factors. The majority of muscle development occurs during

gestation. During this time, animals undergo primary and secondary myogenesis to form muscle fibers, and the total number of muscle fibers an animal will have throughout the entirety of their life is set before birth (Rowe and Goldspink et al., 1969; Beerman et al., 1978; Wigmore and Stickland et al., 1983). After birth, the muscle fibers undergo hypertrophy where they increase in size but not in number (Rowe and Goldspink et al., 1969; Beerman et al., 1978; Wigmore and Stickland et al., 1983; White et al. 2010). Thus, the period of gestation is crucial for proper muscle development, and this tissue is susceptible to programming. Both meat quality, defined by factors such as tenderness, juiciness, and flavor, as well as meay quantity are important factors in marketable animals, and producers are interested in efficiently producing animals with the greatest amounts of high quality meat. The muscle itself is an extremely important tissue to the animal since it serves as an amino acid reservoir and is involved in metabolism (Brown et al., 2014; Wolfe, 2006).

Changes in muscle quantity and quality in response to poor maternal nutrition could be detrimental to production, efficiency, and animal health. Therefore, it is important to understand the factors and mechanisms that contribute to the programming of muscle. Production of healthy and efficient animals is necessary due to the rising demand quality protein sources. By 2050, the global population is expected to increase by 30% and reach 9.7 billion people (United Nations, 2015). Livestock significantly contribute to the global food economy by providing dairy, egg, and meat products. The increase in population will result in an increased demand for animal products (Thorton 2010), and producers will have to meet this demand through more efficient animal production. Since poor maternal nutrition can have detrimental effects on livestock production, and therefore efficiency, it is essential to better understand the effects of programming.

Prenatal Muscle Development

Myoblasts are first recruited from the mesenchyme and differentiate into muscle progenitor cells (MPCs; Pittenger et al., 1999; Biressi et al., 2007). Proliferating MPCs co-express transcription factors Paired box 3 (Pax3) and 7 (Pax7; Biressi et al., 2007). In particular, Pax7 is an early marker of MPCs and is essential in maintaining these cells in a proliferative state (Zammit et al., 2016). Downregulation of Pax7 before differentiation is essential for progression through myogenesis (Zammit et al., 2006; Yablonka-Ruveni et al., 2008). Muscle progenitor cells will differentiate into muscle fibers through appropriate temporal expression of myogenic regulatory factors (MRFs), which drive the processes of proliferation and differentiation. These include myogenic factor 5 (MYF5), myogenic differentiation factor 1 (MyoD), MRF4, and myogenin (Yablonka-Reuveni and Rivera, 1994). Myogenic regulatory factors drive the process of differentiation and are expressed in a sequential order to regulate muscle fiber formation (Berkes and Tapscott, 2005; Braun and Guatel, 2011). These four factors regulate myogenesis to form myotubes which mature into myofibers (Rehfeldt et al., 2004). Specifically, Myf5 and MyoD commit early progenitor cells to the muscle cell type. As differentiation proceeds, MyoD binds to myostatin, contributing to the withdrawal of the myoblast from the cell cycle along with the increased expression of myogenin (Spiller et al., 2002; Singh and Dilworth, 2013). Myogenin and MRF4 later allow myoblasts to fuse and differentiate into muscle fibers (Rehfeldt et al., 2004). Myogenin and MRF4 target proteins that regulate the switch from proliferation to differentiation including retinoblastoma protein (RB), which inhibits cell cycle progression, and Cyclin D1 and cyclin dependent kinase-4 (CDK4), which regulate the cell cycle (Spiller et al., 2002).

Differentiation occurs in several waves, which generate primary and secondary fibers, and supports hypertrophy. The initial stage is primary myogenesis, where MPCs migrate and fuse before innervation, forming primary myofibers (Beerman et al., 1978). The second stage is termed secondary myogenesis and is dependent on innervation. Secondary fibers are derived from MPCs, which are originally maintained in a proliferating and undifferentiated state (Beerman et al., 1978). These MPCs then differentiate into myoblasts and fuse adjacent to primary myofibers to form secondary myofibers (Beerman et al., 1978). Myogenesis in sheep begins around d 20 of gestation and is completed between d 80 and 125 depending on the muscle (Ashmore et al., 1972, Fahey et al., 2005). Since all of the muscle fibers in the fetus are formed during gestation, there is no net increase in fiber number after birth, only fiber size (Rowe and Goldspink et al., 1969; Glore and Layman, 1983; Wigmore and Stickland et al., 1983; Nissen et al., 2003; Zhu et al., 2006; Rehfeldt et al., 2011).

Establishment of skeletal muscle fiber type occurs during gestation (Phillips et al., 1986; Condon et al., 1990; Pette and Staron, 2001). Primary myofibers mainly express slow myosin heavy chain isoforms whereas secondary myofibers express fast myosin heavy chain isoforms which mature into Type II fibers (Pin et al., 2002). Fiber type determination occurs at the myoblast stage before fusion and myofiber formation. Different factors including myoblast lineage and innervation determine the muscle fiber type (DiMario and Stockdale, 1997). However, fiber types can switch due to various factors such as changes in insulin metabolism (Ryder et al., 2003), hormones (Staron et al., 2000), exercise (Henriksson 1992; Booth and Baldwin 1996), or the natural switch from embryonic to adult phenotypes (Whalen et al., 1984; Agbulut et al., 2003). This means that skeletal muscle fibers are capable of responding to altered functional and metabolic demands by changing their fiber type (Pette and Staron, 2001).

Postnatal Muscle Growth

Muscle fibers continue to increase in size postnatally until maturity. During prenatal myogenesis, a portion of myogenic precursor cells remain undifferentiated in a quiescent state adjacent to the myofibers, allowing them to contribute to postnatal growth (Hawke and Garry, 2001). At birth, these MPCs, termed satellite cells, account for up to 30% of total myofiber nuclei (Yin et al., 2013). In postnatal animals, satellite cells compose approximately 3 to 6% of total muscle nuclei (Yin et al., 2013). The increased satellite cell populations in early life contribute to early postnatal hypertrophy and become incorporated into the growing myofiber (Moss et al., 1971). Satellite cells are mononucleated and located between the basal lamina and the sarcolemma in the muscle (Moss and Leblond, 1971). Satellite cells possess their own plasma membrane, separate from membrane of the myofibers (Scharner et al., 2011). Satellite cells are distinct from general muscle nuclei, and distinguished by specific markers such as the transcription marker Pax7. They can also be identified by surface markers such as N-CAM, M-cadherin, and CD34 (Biressi et al., 2010). In mature animals, the satellite cells are usually quiescent except for times of injury, growth, or repair (Aloisi et al., 1973). Satellite cells are activated by several factors such as growth factors, production of sphingosine-1-phosphate, and mitogens (Bischoff, 1986; Kästner et al., 2000; Clemmons, 2009). When satellite cells are activated, their genetic expression changes, allowing for the upregulation of MRfs such as MyoD and myogenin (Pallafacchina et al., 2010). Satellite cells contribute to muscle mass by undergoing asymmetric division. In this process, a portion of the cells differentiate into myoblasts and contribute to the growing muscle fiber. The other portion of the population consists of undifferentiated cells which can replenish the quiescent satellite cell population (Biressi et al., 2010).

Factors Regulating Muscle Development

Hormones and Growth Factors

Hormones and growth factors have important roles in muscle development. For example, growth hormone (GH), in conjunction with insulin-like growth factor 1 (IGF-1), regulates postnatal muscle growth by increasing muscle mass and decreasing adiposity (Velloso et al., 2008). The liver is primarily responsible for IGF-1 production, which is stimulated by GH release. For example, GH administration upregulated IGF-1 mRNA as well as protein in the liver (Matthews et al., 1986). There are also six IGF binding proteins (IGFBPs) that help modulate IGF activity in the tissues (Parker et al., 1998). When the GH/IGF axis is impaired, muscle growth is hindered (Baum et al., 1996; Sotiropoulos et al., 2006; Mavalli et al., 2010). Research demonstrates that when mice are lacking a GH receptor, they have decreased muscle mass and improper growth of myofibers (Baum et al., 1996; Sotiropoulos et al., 2006). The mice lacking the GH receptor also exhibit a reduction in circulating IGF-1. (Sotiropoulos et al., 2006). In vitro, GH stimulates the proliferation of MPCs and fusion of myoblasts via the activation of IGF-1 (Mavalli et al., 2010). Other hormones such as testosterone can influence muscle hypertrophy, whereas increased concentrations of corticosterone may cause atrophy (Crowley and Matt et al., 1996). Growth factors, such as fibroblast growth factor (FGF), can also affect muscle growth and development. During gestation, the FGF family is expressed in the mesoderm, where FGFs have a vital role in the outgrowth of limbs (Olwin et al., 1994). The FGF family of proteins also is involved in regulating the balance between proliferation and differentiation of MPCs by activating MyoD (Hauschka et al., 1992).

Amino Acids and the mTOR Pathway

A large portion of muscle hypertrophy takes place during late prenatal and early postnatal life, when fibers are rapidly growing, but this process continues throughout adulthood (Davis and Fiorotto, 2009). Protein accretion contributes to muscle hypertrophy and occurs during gestation and in postnatal growth. Protein accretion occurs when the rate of protein synthesis exceeds the rate of protein degradation (Schiaffano et al., 2013). Different factors regulate protein synthesis in the early neonatal life, but it is largely controlled by insulin signaling and amino acid sensing (Davis and Fiorotto, 2009). Circulating concentrations of insulin, amino acids, and glucose change in response to diet. Each of these factors stimulate skeletal muscle protein synthesis in the neonate (Davis and Fiorotto, 2009). Studies performed *in vivo* and *in vitro* demonstrate that insulin stimulates protein synthesis in the skeletal muscle and whole body (Liechty et al., 1992; Kimball et al., 1998; Wester et al., 2000; Brown and Hay, 2006).

Amino acids, especially leucine, serve as a signal for protein synthesis and are also precursors for proteins (Kimball and Jefferson, 2006). A postprandial increase in leucine, but not valine or isoleucine alone, stimulated muscle protein synthesis in neonatal pigs. However, the response to leucine was less than the synthesis response to a complete amino acid mixture (Escobar et al., 2005; 2006). The leucine-mediated response in protein synthesis involves the activation of the mammalian target of rapomycin (mTOR) pathway, and protein synthesis is partially regulated by mTOR. The mTOR complex 1 (mTORC1) is regulated by growth factors, energy status, oxygen, and amino acid concentrations and contributes to protein synthesis, lipid synthesis, and autophagy (Laplante and Sabatini, 2012). Specifically, amino acids, such as leucine and arginine, act as upstream signals to activate mTORC1 (Blommaart et al., 1995; Hara et al., 1998). Protein synthesis is directly regulated by mTORC1 since it phosphorylates the translational regulators eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4E-

BP1) and S6 kinase 1 (S6K1), which then promote protein synthesis (Ma and Blenis, 2009). In addition to regulating muscle protein synthesis, mTORC1 is involved in the synthesis of lipids to generate membranes, and also positively regulates cellular energy metabolism and ATP production (Laplante and Sabatini, 2012).

Muscle Metabolism

Muscle and Inter-Organ Metabolism

Skeletal muscle plays an integral role in metabolism and inter-organ crosstalk since it is involved with glucose and protein metabolism (Meyer et al., 2002). Skeletal muscle comprises 40 to 50% of body mass, making it the most abundant insulin-sensitive tissue (Baron et al., 1988; DeFronzo et al., 1985; Zurlo et al., 1990). Additionally, skeletal muscle is responsible for 75 to 95% of insulin mediated glucose disposal and is responsible for 20 to 30% of oxygen consumption (DeFronzo et al., 1985; Baron et al., 1988; Zurlo et al., 1990). Skeletal muscle also serves as an amino acid reservoir that sustains protein synthesis within the muscle and whole body (Wolfe, 2006). After a meal, approximately 40% of glucose is taken up by the muscle for storage as glycogen (Meyer et al., 2002). Glycogen storage facilitates rapid energy production for fiber contraction even when glucose is not available from the diet (Argiles et al., 2016). As stores of glycogen become depleted, increasing amounts of energy are generated through the process of gluconeogenesis (Shrayyef and Gerich, 2010). Muscle is essential for this process since during times of fasting, protein within muscle can be catabolized to provide energy substrates and essential amino acids (Frontera and Ochala, 2015). The regulation of glucose is essential for energy balance within the muscle and whole body. In the cytoplasm of cells, including skeletal muscle, the process of glycolysis generates energy substrates for ATP

production from glucose (Argiles et al., 2016). The liver and kidney also store glycogen, and glucose regulation by these three organs allows the body access to energy substrates. Interactions between the liver and muscle regulate carbohydrate metabolism and are largely responsible for achieving energy balance in the fed- and fasted-state (Argiles et al., 2016). Skeletal muscle is vital to metabolism and overall health since any changes to muscle mass, metabolic rate, hormone concentrations, or other circulating factors could significantly alter utilization of energy within the body.

Fiber Type Metabolism

Muscle metabolism is dictated by the characterization and distribution of fibers within that muscle (Scott et al., 2001). Muscle fiber types are classified based on their speed of contraction as well as their different need for energy substrates (Scott et al., 2001; Biressi et al., 2007). Muscle fibers are composed of fibrillary proteins including actin and myosin, and different muscle fiber types have been classified based on morphological differences of these filaments. There are three main types of muscle fibers that contribute to skeletal muscle composition, although variations do occur (Scott et al., 2001). The main classifications are type I or slow-twitch oxidative, type IIa or fast-twitch oxidative, and type IIb or fast-twitch glycolytic (Pette et al. 2001). The terms “fast” and “slow” twitch refer to the speed of ATP hydrolysis and therefore, contraction within the fiber. The slow muscles have an increased myoglobin content which contributes to their darker red color, whereas the fast muscles have a reduced myoglobin concentration and appear white or lighter in color (McComas et al., 1996). The greater myoglobin content allows the slow muscle fibers to have a greater oxidative capacity and therefore rely on oxidative metabolism (McComas et al., 1996). Oxidative metabolism occurs

under aerobic conditions and produces 36 ATP per molecule of glucose. Additionally, slow twitch fibers have a greater density of capillaries and oxidative enzymes, making them more resistant to fatigue than fast twitch fiber types (Matsakas et al., 2009). Alternatively, the fast twitch type utilizes anaerobic metabolism, primarily glycolysis, to generate ATP (McComas et al., 1996). Glycolysis produces only two ATP per glucose molecule. This means that fast twitch muscles can contract more readily, but fatigue quicker than slow twitch muscles (Matsakas et al., 2009). The differences in fiber types account for the different movements. Slow-twitch muscles are necessary for endurance exercise, whereas fast-twitch muscles are necessary for quick and powerful bursts of movement (Morrison et al., 1970). Fiber types are not permanently fixed during gestation and throughout postnatal life. Muscle fibers can adapt to altered metabolic states, which in turn alters fiber type distribution and composition (Daniel et al., 2007; Maltin, 2008; Schiffano et al., 2013). These changes to composition can then influence muscle metabolism as well as whole body metabolism.

Poor Maternal Nutrition

Both under- and over-feeding during gestation can negatively affect the offspring and impact both prenatal and postnatal muscle development (Ford et al., 2007). Although there is an increase in net muscle mass after birth due to hypertrophy, all of the muscle fibers are formed during gestation (Rowe and Goldspink et al., 1969; Beerman et al., 1978; Wigmore and Stickland et al., 1983; White et al. 2010). This makes muscle tissue particularly vulnerable to intrauterine conditions, maternal nutrition, and other factors that can influence muscle development (Zhu et al., 2006). Skeletal muscle is particularly susceptible to fetal programming because it is a lower priority tissue. Nutrients are first partitioned to organs such as the brain,

liver, and heart before muscle (Tchirikov et al., 1998; Yajnik, 2004). Therefore, to maintain vital organs, any nutritional insult to the mother during gestation can greatly impact muscle formation (Zhu et al., 2006).

Nutrient Restriction

Reduced feed intake during gestation can impact body and muscle composition as well as muscle fiber development. For example, dams who faced reduced nutrient intake during pregnancy produced lambs that were overall fatter and had reduced lean-to-fat ratios (Tygesen et al., 2007). A study conducted at the University of Connecticut demonstrated that in the semitendinosus muscle, lipid content was 92.5% greater in offspring from restricted animals compared with CON at birth. At 3 mo of age, restricted offspring showed a 23.6% decrease in lipid accumulation in the muscle compared with controls (Reed et al., 2014). This demonstrates that restricted-feeding alters lipid accumulation in the semitendinosus muscle and therefore muscle composition.

Nutrient restriction can alter the total number of muscle fibers as well as the distribution of primary and secondary fibers. A study conducted by Zhu et al., (2004), found that a 50% NRC nutrient restriction in pregnant ewes from d 28 to 78 of gestation reduced the total number of secondary muscle fibers and also the ratio of primary to secondary muscle fibers in lambs (Zhu et al., 2004). These results indicate that fiber development is impaired in response to maternal restricted-feeding. Another study by Zhu et al. (2006) demonstrated that 8-mo-old lambs born to nutrient deficient mothers had fewer total muscle fibers than control animals (Zhu et al., 2006). In addition to impacting the number of fibers within the muscle, nutrient restriction can also alter the cross-sectional area (CSA) of muscle fibers. Underfeeding ewes during gestation impacted

offspring muscle CSA. For example, at birth and 3 mo of age, offspring from restricted-fed mothers weighed the same as offspring from control-fed mothers, but CSA was different (Reed et al., 2014). CSA of semitendinosus muscle fibers was 57% greater in restricted animals compared with control (Reed et al., 2014). However, at 3 mo of age, the muscle fiber CSA of the restricted lambs was reduced by 15% compared with controls (Reed et al., 2014). Maternal nutrient restriction during gestation similarly impairs cattle skeletal muscle, as demonstrated in a study conducted by Gonzalez et al. (2013). The fetal infraspinatus (INF) muscle was measured in calves from heifers fed 60% or 100% NRC from conception to either d 85 or 140 of gestation. At d 85 of gestation, the restricted offspring had increased muscle fiber CSA compared with the control group. At d 140, the restricted calves had reduced fiber CSA in the INF muscle (Gonzalez et al., 2013).

Maternal nutrient-restriction can alter genetic expression of myogenic regulatory factors of the offspring. Even moderate maternal undernutrition negatively alters fetal muscle development by impacting the expression of myogenic regulatory factors in the muscle (Zou et al., 2016). Sows fed a low energy diet from d 1 to 90 of gestation had offspring with reduced fetal weights and decreased muscle fiber density. The low energy group had reduced mRNA expression of myogenin, MRF4, and creatine kinase compared with the control group at both d 55 and 90 of gestation in the fetal pig (Zou et al., 2016). Further, restricted maternal diet can alter genes associated with muscle cell proliferation and signal transduction (Hoffman et al., 2016). These studies demonstrate that genes involved in myogenesis are altered due to poor maternal nutrition, which may result in changes to the muscle phenotype in offspring of restricted-fed dams.

Nutrient Excess

Maternal over-nutrition during gestation also alters skeletal muscle development. Myogenesis and adipogenesis occur around the same time during gestation, and excess nutrients can affect both muscle and fat formation (Tong et al., 2009). Maternal over-feeding during gestation can result in increased adipogenesis in the muscle and in the carcass as a whole, changing composition (Tong et al., 2009). Sows who were over-fed from conception to d 50 of gestation had piglets with increased adiposity in the muscle at birth (Daniel et al., 2007). Huang et al. (2012) also showed that maternal obesity during gestation produced offspring with increased collagen and cross-linking in the LD and semitendinosus muscle (Huang et al., 2012). The excess collagen and crosslinking reduces meat tenderness and makes it less palatable to the consumer. Further, sows who faced maternal over-nutrition during mid-gestation had offspring with impaired fiber development and reduced carcass quality (Cerisuelo et al., 2014). Lipid content in the semitendinosus muscle was 212.4% greater in offspring from over-fed ewes compared with the controls at birth. At 3 mo of age the over-fed animals had 36.1% greater lipid accumulation than control animals (Reed et al., 2014). These changes to composition could negatively impact muscle growth and metabolism.

Maternal over-nutrition can also impact muscle fiber number and fiber distribution. Sows who were over-fed during gestation had piglets with reduced total, primary, and secondary fibers, demonstrating that over-feeding during gestation influences prenatal muscle fiber hyperplasia (Cerisuelo et al., 2014). Fiber diameter can also be reduced by maternal obesity. Specifically, at birth, lambs from obese ewes had reduced semitendinosus muscle fiber diameter compared with controls (Tong et al., 2009). Further, increased maternal nutrition during gestation altered skeletal muscle development in ovine offspring. From birth to 3 mo of age, the

lambs from over-fed mothers weighed 13% more than control lambs. At birth, CSA of muscle fibers in the semitendinosus was 47% greater in offspring of over-fed animals compared with controls. At 3 mo of age, the CSA for the offspring of over-fed dams was reduced by 17% (Reed et al., 2014). Thus, maternal over-nutrition during gestation impacts myogenesis, resulting in changes to muscle fiber number, fiber type distribution, and CSA.

Maternal over-nutrition can also influence gene expression within the muscle. In cattle, maternal over-nutrition increased mRNA expression of adipogenic markers in skeletal muscle (Duarte et al., 2014). A study in sheep demonstrated that maternal obesity impaired myogenesis in the semitendinosus by downregulating myogenic markers including MyoD, myogenin and desmin in offspring from obese mothers compared with controls at birth (Tong et al., 2009). Additionally, maternal over-nutrition altered genes in offspring muscle related to protein synthesis, growth, and metabolism (Hoffman et al., 2016). Changes to gene expression can impact the skeletal muscle and demonstrate that maternal over-nutrition causes negative programming effects. The negative effects of maternal over-nutrition, including changes to composition, fiber number, fiber size, and gene expression can impact postnatal muscle growth and metabolism.

Poor Maternal Nutrition and Stem Cell Function

Poor maternal nutrition during gestation also alters stem cell populations in the offspring, specifically, satellite cells and mesenchymal stem cells. In mice, Woo et al. (2011) determined that offspring from restricted-fed mothers had altered muscle composition as well as changes in satellite cell numbers. Offspring of underfed dams had reduced overall muscle mass, but similar birth weights to control animals (Woo et al., 2011). Further, satellite cell numbers were reduced

by 33% in offspring of restricted-fed dams. Satellite cell function was examined later at 6 wk of age, since these muscle progenitor cells are essential for muscle repair and hypertrophy. During muscle regeneration, the number of regenerating fibers was reduced by 32% 3 d after injury in offspring of restricted-fed dams (Woo et al., 2011). Gonzalez et al. (2013) used cattle to demonstrated that a maternal restricted diet (60% of NRC requirements) reduced the number of Pax7 positive nuclei in offspring INF by almost 20% at d 85 of gestation, suggesting a loss of muscle progenitor cells. Additionally, offspring satellite cell function and subsequent hypertrophy is hindered by maternal restricted feeding (Raja et al., 2016). Satellite cells are essential for postnatal muscle hypertrophy and muscle repair. Therefore, any insults during gestation can have lasting impacts of muscle growth. The results of these studies indicate that satellite cell populations and function are vulnerable to fetal programming.

Mesenchymal stem cells are multipotent stem cells that contribute to the development of tissues including adipose, bone, and muscle. As part of the bone marrow niche, MSC help maintain and repair these tissues throughout adult life. MSC are responsive to changes in hormone and metabolite concentrations, and therefore susceptible to programming. Changes to maternal diet can cause MSC to favor differentiation into one cell lineage over another (Devlin and Bouxsein, 2012). Additionally, poor maternal nutrition, both restricted- and over-feeding, altered proliferation and metabolism of MSC (Pillai et al, 2016). Maternal restricted- and over-feeding reduced MSC proliferation by 51% and 60%, respectively (Pillai et al., 2016). Mitochondrial basal respiration, ATP production, and maximal respiration were impacted by over- and under-feeding, indicating changes to the metabolism of the MSC due to maternal programming (Pillai et al., 2016). Evidence supports that early perturbations to stem cell populations can have-long lasting impacts on muscle growth and development (Woo et al.,

2011). Additionally, changes to these stem cell populations may provide insight into some of the mechanisms by which programming alters muscle metabolism and function.

Effects of Poor Maternal Nutrition on Metabolism

Whole body metabolism

Poor maternal nutrition can cause changes to whole body metabolism, ultimately affecting muscle. Specifically, poor maternal nutrition alters the intrauterine environment and concentrations of offspring hormones and growth factors, thereby altering metabolism. For example, over- and under-feeding ewes during gestation resulted in lambs with decreased serum concentrations of IGF1 and IGF binding protein 3 at birth (Hoffman et al., 2014). Additionally, at 3 mo of age, offspring from over-fed ewes had increased leptin concentrations compared with restricted-fed offspring (Hoffman et al., 2016). In the same study, average insulin concentrations were increased over time in offspring from over-fed and restricted-fed ewes compared with controls (Hoffman et al., 2016). Maternal under-nutrition also alters glucose tolerance in the offspring (Ford et al., 2007). Lambs born to restricted-fed ewes exhibited increased area under the curve for glucose and insulin at d 63 of age compared with control (Ford et al., 2007). Further, adult offspring of obese ewes had increased plasma glucose, insulin, and leptin concentrations after a twelve-week feeding challenge (Long et al., 2010). Early maternal under-nutrition also results in altered metabolism. Specifically, maternal under-feeding resulted in reduced insulin sensitivity and increased insulin secretion in adult offspring (George et al., 2011). Another study found that in sheep, early gestational under-nutrition caused a reduction in hepatic expression of gluconeogenic factors as well as reduced insulin sensitivity in the adipose tissue (Poore et al., 2014). These changes to hormones, growth factors, and metabolism could be

mechanisms by which metabolism is programmed in response to poor maternal nutrition during gestation.

Muscle Metabolism and Oxidative Stress

Not only does poor maternal nutrition affect whole body metabolism through changes to hormones and growth factor concentrations, there are specific programming effects on muscle metabolism and mitochondrial function. In rats, maternal obesity resulted in offspring with reduced skeletal muscle glucose transporter 4 (GLUT4) (Simar et al., 2012). There was also increased monocarboxylate transporter 1 (MCT1) protein (Simar et al., 2012). When these offspring continued on an obesogenic diet post-weaning, there were continued changes to the muscle. At 18 weeks of age, the offspring continued to have reduced muscle GLUT4 concentrations, as well as decreased mRNA expression of carnitine palmitoyl transferase-1, and MyoD and myogenin protein expression (Simar et al., 2012). These findings demonstrate that maternal obesity could be influencing glucose metabolism and muscle specific gene expression.

Poor maternal nutrition can alter mitochondrial function within skeletal muscle cells. Mitochondria generate ATP and are essential for cellular energy production and the regulation of cellular energy metabolism (Hanson, 1989). Both over- and restricted-feeding can alter offspring mitochondria. A study in mice demonstrated that male offspring from obese mothers had a decrease in mitochondrial complex II-III as well as impaired insulin signaling in the skeletal muscle (Shelley et al., 2009). Another in pigs found that in-utero exposure to high energy diets decreased mitochondrial DNA content in skeletal muscle and downregulated mRNA concentrations of genes associated with mitochondrial biogenesis and function (Zou et al., 2017). Additionally, reduced maternal dietary energy intake decreased expression of genes involved in

mitochondrial biogenesis in the muscle, including peroxisome proliferator-activated receptor gamma coactivator 1α (PPARGC1A), nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM), β subunit of mitochondrial H(+)-ATP synthase (ATB5B), sirtuin 1 (Sirt1), and citrate synthase (CS; Zou et al., 2016).

Poor maternal nutrition can affect the fiber type distribution, and therefore skeletal muscle metabolism in the offspring. For example, over-feeding during gestation altered fiber-type proportions in porcine offspring by increasing the number of Type IIA fibers, leading to an increase in oxidative capacity. However there was no change to muscle fiber CSA or total fiber number (Markham et al., 2009). Over-feeding during gestation resulted in offspring that had a 13.3% increase in type IIA fibers at birth (Reed et al., 2014). In contrast, restricted feeding during gestation resulted in offspring that had a 14.6% decrease in type IIA fibers at birth (Reed at al., 2014). Changes to fiber type distribution can have long-lasting impacts on muscle metabolism and can additionally result in changes to reactive oxygen species (ROS) and oxidative stress within the muscle (Jackson, 2005). During muscle contraction, free radicals are generated, resulting in oxidative damage to the tissue if not properly detoxified (Davies et al., 1982). Oxidative stress affects the mitochondria and myofibers of muscle, resulting in damage of the tissue (Davies et al., 1982). Oxidative stress can induce mitochondrial apoptosis (Giorgio et al., 2005). Due to their redox systems, type I fibers are more resistant to oxidative stress than type II fibers (Jackson, 2005). Therefore, an increase in type II fibers could indicate a decreased ability for the offspring to detoxify ROS in the muscle, further impacting growth and metabolism.

Oxidative stress has many negative effects and can impact tissues such as muscle and adipose. The amount of ROS production and oxidative stress is dependent on different factors

such as the muscle fiber type composition and fat content in the muscle (Pinho et al., 2017). Oxidative stress alters satellite cell differentiation, causing more of these cells to join the adipocyte rather than the myogenic lineage (Vettor et al., 2009), resulting in increased adipose within the muscle. Increased oxidative stress is correlated with increased lipid peroxidation, resulting in mitochondrial damage and metabolic disruption (Davies et al., 1982).

Oxidative stress is associated with muscle dysfunction. Studies indicate that mitochondrial uncoupling may be a protective mechanism to reduce ROS (Baynes et al., 1991; MacLellan et al., 2005). This is supported by experiments which demonstrate that mitochondrial uncoupling protein 3 (UCP3) upregulation reduces ROS production and facilitates fatty acid oxidation in L6 myocytes (MacLellan et al., 2005). In patients with type 2 diabetes, plasma hydroperoxides, a marker of oxidative stress, are increased and inversely correlated with glycemic control (Baynes et al., 1991). Other studies demonstrate that low-grade oxidant stress reduces insulin-stimulated glucose transport and insulin signaling in insulin sensitive cells such as L6 myocytes and 3T3-L1 adipocytes (Maddux et al., 2001; Rudich et al., 1997; Rudich et al., 1998). Continuous evidence supports that oxidative stress is associated with impaired insulin action and metabolism in muscle.

Poor maternal nutrition induces oxidative stress in offspring. Malti et al. (2014) demonstrated that mothers who were obese and had nutrient rich diets during gestation had increased oxidative stress. The offspring of these mothers also had increased amounts of oxidative stress, implicating a programming effect of maternal diet (Malti et al., 2014). Another study in humans demonstrated that children of mothers with increased body condition score (BCS) had increased lipid peroxidation and oxidative stress from ages 8 to 13 (Mohn et al., 2007). Restricted maternal nutrition can similarly impact oxidative stress and the mechanisms in

place to modulate it. For example, He et al., (2012) demonstrated that when maternal protein is restricted, offspring had reduced antioxidant capacity. Even after a period of controlled feeding and nutrient recovery, these offspring still had impaired ability to detoxify free radicals, leading to increased oxidative stress (He et al., 2012). Further studies show that redox status is altered by poor maternal nutrition. Specifically, the expression of the antioxidant magnesium dependent superoxide dismutase is increased by maternal diet (Tarry-Adkins et al., 2016). Poor maternal nutrition during gestation may be altering skeletal muscle metabolism by contributing to changes in fiber type metabolism and inducing oxidative stress.

Conclusion

Optimal livestock production is dependent on proper gestational management and efficient offspring growth. As a result of poor maternal nutrition, changes to offspring mRNA and protein expression has the potential to reduce livestock growth rates and lean carcass mass, as well as impact the health of the offspring (Wu et al., 2004). Specifically, both under- and over-feeding can impact muscle formation, offspring metabolism, stem cell populations, and oxidative stress. Muscle is the meat product that consumers buy, and the demand for high quality protein will only increase as the population expands. Often, offspring from mothers who are either under- or over-nourished demonstrate divergent muscle phenotypes from control animals. Alterations to metabolism may be an underlying mechanism that drives these phenotypic changes. Therefore, it is important to investigate how poor maternal nutrition alters offspring muscle metabolism. We hypothesized that over- or restricted-feeding during gestation would alter muscle myoblast metabolism as well as offspring Longissimus dorsi muscle (LD) metabolome.

MATERIALS AND METHODS

Animals

All animal procedures were reviewed and approved by the University of Connecticut Institutional Animal Care and Use Committee (A13-059). Animal procedures were previously reported in Pillai et al. (2017). Multiparous Western White-faced ewes (n = 47), aged 3 years or older were estrus synchronized using a controlled intravaginal drug release device (CIDR; Easi-Breed CIDR Sheep Insert, Zoetis, Parsippany, NJ). The CIDR was inserted intravaginally and removed from each ewe after 12 days. Following this, the ewes were given an intramuscular injection of PGF2α [Lutalyse, 5 mg/mL; Zoetis, Inc.; (Knights et al., 2001)]. The ewes were housed in a group and bred to 1 of 4 related Dorset rams. Day 0 of pregnancy was determined when ewes received a rump mark and showed no further evidence of remarking. Twenty days later, ewes were moved to 3 x 1 m individual pens that allowed visual but not physical contact with other ewes and individually fed. Ewes were transitioned to a complete pelleted feed based on National Research Council (NRC) requirements for ewes gestating with twins, over a 7-day period starting at d 20. Using transabdominal ultrasound, 47 ewes were confirmed pregnant on d 28.5 ± 0.4 (Jones et al., 2016). At d 30, ewes were randomly assigned to a 3 x 4 factorial arrangement of treatment structure with main effects of diet (3) and time point of gestation (4; *n* = 5 to 7 ewes per treatment). Pregnant ewes were fed either a control-fed (CON; 100% NRC; *n* = 27), restricted-fed (RES; 60% NRC; *n* = 28), or over-fed diet (OVER; 140% NRC; *n* = 27) starting at d 30.2 ± 0.2 of gestation based on the NRC requirement for TDN (National Research Council, 1985). Based on ewe body weight, treatment diets (Central Connecticut Farmer's COOP, Manchester, CT) of 60, 100, or 140% NRC were achieved by offering differing amounts of complete pelleted feed (Pillai et al., 2017). For each grain delivery (*n* = 3), analysis of

nutrients was completed by Dairy One (Ithaca, NY). Blocks containing sodium chloride and fresh water were freely available to the ewes. At the beginning of treatment and weekly thereafter, ewes were weighed and body condition score (BCS) was independently evaluated on a scale of 1-5 by 2 trained observers. Offspring born to control-, restricted-, or over-fed ewes are denoted as CON, RES and OVER, respectively.

Sample Collection

At d 90 and 135 of gestation, ewes (*n* = 5 to 7 per treatment combination) were weighed and euthanized. An intravenous injection of Beuthanasia-D Special (Merck Animal Health; Summit, NJ) containing 390 mg/mL sodium pentobarbitol and 50 mg/mL phenytoin based on body weight was given to each ewe. Following the injection, each ewe was exsanguinated as previously detailed (Reed et al., 2014). The fetuses were removed following a hysterectomy for necropsy and tissue analysis to evaluate the effects of restricted- and over-feeding on offspring development during gestation. A subset of ewes were allowed to undergo parturition (birth; *n* = 5 to 7 per dietary treatment). Lambs were allowed to nurse colostrum for up to 24 hours after birth. Lambs were offered colostrum replacer (4 ewes total: 2 twin control litters and 2 twin restricted litters; DuMor Blue Ribbon Multi-Species Colostrum Supplement; Distributed by Tractor Supply Company, Brentwood, TN) if the ewe did not produce sufficient colostrum. Within 24 hours of birth, lambs were weighed and euthanized with an i.v. overdose of Beuthanasia-D Special (390 ng/mL Sodium Pentobarbital and 50 mg/mL Phenytoin based on BW) and exsanguinated.

Due to reasons unrelated to the study, four ewes did not complete the experiment. Five offspring were not viable and therefore not included in the experiment (2 mummified and 3 stillborn). For the purposes of these experiments, a total of 72 offspring (n = 8 per treatment per

time point) from 47 ewes at d 90 and 135, and within 24 hours of birth were included in the final analysis.

Muscle samples from each offspring at d 90, 135, and birth were collected from the LD immediately after euthanasia and samples were immediately snap frozen in liquid nitrogen. Samples were stored at −80°C until further use. Whole blood was obtained from live lambs at the birth time point via jugular venipuncture and processed for serum within 24 hours of parturition.

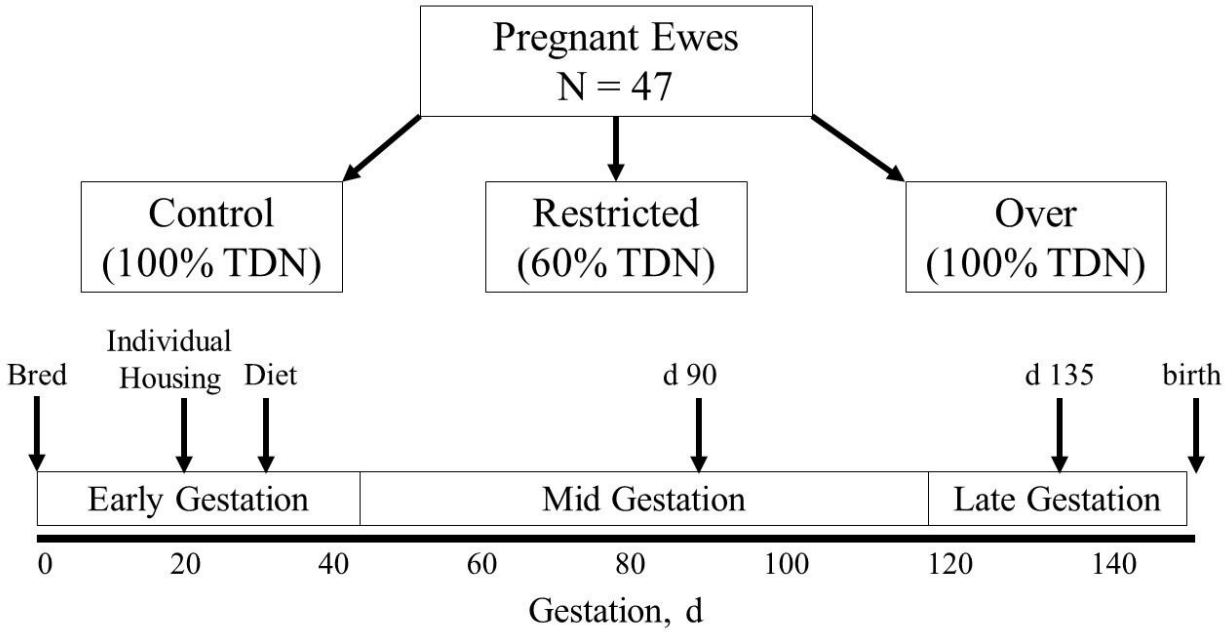


Figure 1. Experimental design, adapted from Pillai et al. (2017). To evaluate the effects of restricted- or over-feeding on offspring development during gestation, pregnant ewes (n = 82) were housed in individual pens on d 20 of gestation (Individual housing) and randomly assigned into a 3 × 4 factorial arrangement of treatments which began at d 30 of gestation (Diet). The treatment structure included 3 dietary levels (CON: 100% TDN; RES: 60% TDN; OVER: 140% TDN) and 4 time points during gestation (n = 5 to 7 ewes per treatment combination). At d 90 or 135 of gestation, ewes were euthanized and the fetus(es) were removed for necropsy and tissue analysis. A third group of ewes underwent parturition (birth), after which lambs were necropsied within 24 h of parturition.

C2C12 Cell Culture

Proliferation

C2C12 cells (ATCC CRL-1772, Manassas, VA) were cultured in growth media containing Dulbecco's modified Eagle medium (DMEM) with 4 g/L D-glucose (Gibco Laboratories, Gaithersburg, MD) and 10% fetal bovine serum (FBS), 1% penicillin/ streptomycin, 0.3% fungizone, and 0.2% gentamycin. Cells were passed once and plated on 24-well plates at a density of 3,000 cells per well. Cells were allowed to attach and proliferate in growth media for 24 hours. After 24 hours, the media was changed so that the growth media contained 10% offspring serum instead of FBS. Serum collected from CON (n = 8), RES (n = 8), and OVER (n = 8) lambs at birth was added to media in duplicate wells. Each plate contained two wells cultured in growth media with FBS to serve as a plate control. Following the manufacturer's instructions, after 48 hours, the cells were pulsed with 5-bromo-2'-deoxyuridine (BrdU) for 30 minutes (Click-iT EdU Alexa Fluor 488 Imaging Kit, Invitrogen, Carlsbad, CA), fixed with 4% paraformaldehyde, and immunostained to measure the percentage of cells in S-phase. Cells were incubated with Hoescht 33342 for 1 hour to visualize nuclei. The cells were imaged using a Zeiss Observer, and 5 random images per well were taken. The percentage of proliferating cells was determined by the number of BrdU(+) cells divided by the total number of nuclei and multiplied by 100. Each experiment was replicated once.

Differentiation

C2C12 cells were cultured in growth media containing DMEM with 4 g/L D-glucose, 10% FBS, 1 % penicillin/ streptomycin, 0.3% fungizone, and 0.2% gentamycin. Cells were passed once and plated onto 24-well plates at a density of 25,000 cells per well. Cells were

allowed to attach and proliferate in growth media for 72 hours to reach 100% confluency. After 72 hours, the media was changed to differentiation media containing 2% offspring serum, 1% penicillin/ streptomycin, 0.3% fungizone, and 0.2% gentamycin. Serum from CON (n = 8), RES (n = 8), and OVER (n = 8) lambs was added to media in duplicate wells. C2C12 cells were cultured in their individual media and allowed to differentiate in treatment media for 5 days. Each plate contained two wells differentiated in differentiation media containing horse serum to serve as a plate control. To determine the effects of offspring serum on differentiation, myotubes were fixed with 4% paraformaldehyde and immunostained for myosin heavy chain (MyHC; Appendix 1). Hoescht 33342 was used to visualize nuclei. The cells were imaged using a Zeiss Observer, and 5 random images per well were taken. Fusion index was calculated as the number of nuclei within multinucleated MyHC-positive myotubes divided by the number of total number of nuclei and multiplied by 100. Fiber diameter was calculated by determining the average width (diameter measured in 3 places) of MyHC-positive myofibers in each image. Each experiment was replicated once.

Mitochondrial and Glycolytic Stress Assays

Mitochondrial and glycolytic stress were evaluated using the Seahorse XFe24 Extracellular Flux Analyzer (Seahorse Bioscience; North Billercia, MA) following manufacturer's instructions and protocol. In brief, C2C12 cells were plated onto XFe24 culture plates at a density of 15,000 cells per well and cultured in 10% offspring serum in growth media [CON (n = 6), RES (n = 6), or OVER (n = 6)] for 24 hours. Twelve hours before the assay, the XFe24 sensor cartridges were hydrated with Seahorse Bioscience XFe24 Calibrant (pH 7.4) and stored at 37°C.

For the Cell Mito Stress Test Assay (Appendix II), 1 mM pyruvate (Sigma-Aldrich, St Louis, MO), 2 mM glutamine (Sigma-Aldrich), and 10 mM glucose (Sigma-Aldrich) were added to the assay medium, warmed to 37°C, pH was adjusted to 7.4 with 0.1 N NaOH, and the media was sterile filtered. Oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and rotenone/antimycinA were re-suspended with 630 µL, 720 µL, and 540 µL of prepared assay medium, respectively. The injection ports were loaded using the constant volume method described in the manufacturer's protocol. The maintenance medium was removed with a pipette, and each well was rinsed twice with assay media. Assay media (525 µL) was added to each well, and the cell culture plate was incubated at 37°C without CO_2 for 60 minutes. The plate was then loaded into the Seahorse XFe24 Extracellular Flux Analyzer and the standard operating procedure for Cell Mito Stress Test Assay for the machine was followed according to manufacturer's protocol to measure oxygen consumption rate (OCR).

For the Glycolysis Stress Test Assay (Appendix III), 2 mM glutamine was added to Seahorse XF Assay Media (Seahorse Bioscience). The media was warmed to 37°C, pH was adjusted to 7.4 with 0.1 N NaOH, and the media was filter sterilized. The injection ports were loaded using the constant volume method described in the manufacturer's protocol. The maintenance medium was removed with a pipette, and each well was rinsed twice with assay medium. Assay media (525 µL) was added to each well, and the cell culture plate was incubated at 37°C without CO_2 for 60 minutes. The plate was then loaded into the Seahorse XFe24 Extracellular Flux Analyzer. Standard operating procedure for Glycolysis Stress Test Assay for the machine was followed according to manufacturer's protocol to measure extracellular acidification rate (ECAR).

The DNA content in individual wells on the XFe24 cell culture plates was quantified to account for variations in cell density using Macharey-Nagel NucleoSpin Tissue kits (Macharey-Nagel Inc, Bethlehem, PA, USA), according to manufacturer's protocol. The OCR and ECAR measurements were adjusted for total DNA content in each well.

Metabolome Analysis

Sample Preparation

Longissimus dorsi samples (200 ng; n = 8 per treatment per time point) were shipped on dry ice to Metabolon, Inc. (Morrisville, NC) for metabolome analysis. Samples were prepared using the automated MicroLab STAR® system (Hamilton Company). Several recovery standards were added prior to the first step in the extraction process for quality control (QC) purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000, SPEX Sample Prep, Metuchen, NJ) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/ Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by Hydrophilic Interaction Chromatography (HILIC)/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Samples were placed briefly on a TurboVap® (Zymark, Hopkinton, MA) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for analysis.

Quality Assurance/ Quality Control

Several types of controls were analyzed in concert with the experimental samples: a pooled matrix sample generated by taking a small volume of each experimental sample served as a technical replicate throughout the data set; extracted water samples served as process blanks; and a cocktail of QC standards that were carefully chosen not to interfere with the measurement of endogenous compounds were spiked into every analyzed sample allowed instrument performance monitoring and aided chromatographic alignment. Instrument variability was determined by calculating the median relative standard deviation (RSD) for the standards that were added to each sample prior to injection into the mass spectrometers. Overall process variability was determined by calculating the median RSD for all endogenous metabolites (i.e., non-instrument standards) present in 100% of the pooled matrix samples. Experimental samples were randomized across the platform run with QC samples spaced evenly among the injections.

Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectroscopy (UPLC-MS/MS)

All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer operated at 35,000 mass resolution. The sample extract was dried then reconstituted in solvents compatible to each of the four methods. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for more hydrophilic compounds. In this method, the extract was gradient eluted from a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed

using acidic positive ion conditions; however it was chromatographically optimized for more hydrophobic compounds. In this method, the extract was gradient eluted from the same afore mentioned C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. Another aliquot was analyzed using basic negative ion optimized conditions using a separate dedicated C18 column. The basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. The fourth aliquot was analyzed via negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The Mass Spec (MS) analysis alternated between MS and data-dependent sequential mass spectrometry (MSn) scans using dynamic exclusion. The scan range varied slighted between methods but covered 70-1000 m/z.

Data Extraction and Compound Identification

Raw data were extracted, peak-identified and QC processed using Metabolon's hardware and software. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. Metabolon maintains a library based on authenticated standards that contains the retention time/index (RI), mass to charge ratio (*m/z)*, and chromatographic data (including MS/MS spectral data) on all molecules present in the library. Furthermore, biochemical identifications are based on three criteria: retention index within a narrow RI window of the proposed identification, accurate mass match to the library +/- 10 ppm, and the MS/MS forward and reverse scores between the experimental data and authentic standards. The MS/MS scores are based on a comparison of the ions present in the experimental spectrum to the

ions present in the library spectrum. While there may be similarities between these molecules based on one of these factors, the use of all three data points can be utilized to distinguish and differentiate compounds. More than 3,300 commercially available purified standard compounds have been acquired and registered into the laboratory information management system (LIMS) for analysis on all Metabolon platforms for determination of their analytical characteristics.

Statistical Analysis

Cell Culture Data

All data were analyzed using PROC MIXED (SAS Inst. Inc., Cary, NC; version 9.4). Main effects of treatment, gender, and their interaction were analyzed and differences between means were determined using the pdiff statement in LSMEANS. Data are reported as mean ± SE. Main effects are discussed in the absence of a treatment by gender interaction. Significance is tested at $P \leq 0.05$.

Metabolome Data

Data were analyzed using one-way ANOVA, two-way ANOVA, two-way repeated measures ANOVA, and correlation analysis to obtain *P* and *q* values. Statistical significance is declared for *P*-values less than 0.05. A principal components analysis was performed to find hierarchical clusterings of the data. Each principal component is a linear combination of every metabolite and the principal components are uncorrelated. The number of principal components is equal to the number of observations.

RESULTS

Offspring serum alters C2C12 myoblast metabolism but not proliferation or differentiation

Serum collected from offspring born to over- and restricted-fed ewes had no effect on BrDU incorporation (Figure 1) compared with CON ($P \geq 0.39$; CON = 24.89 ± 3.03%, RES = 24.06 ± 3.03%, OVER = 27.74 ± 3.03%). Additionally, serum collected from OVER and RES offspring had no effect on myofiber fusion index (Figure 2A; $P \geq 0.15$; CON = 15.70 ± 2.87%, RES = 9.55 ± 3.23%, OVER = 10.97 ± 3.03%) or myofiber diameter (Figure 2B; $P \geq 0.70$; CON = 15.38 ± 1.33 μm, RES = 14.88 ± 1.46 μm, OVER = 15.25 ± 1.38 μm) compared with CON.

Serum from RES offspring increased proton leak ($P = 0.01$; Figure 3, CON = 14.98 ± 1.81, RES = 22.33 ± 1.81 pmol $O_2 \cdot min^{-1} \cdot \mu g^{-1}$ DNA), but serum from OVER offspring had no effect on proton leak ($P = 0.57$.; OVER = 16.45 ± 1.81 pmol $O_2 \cdot min^{-1} \cdot \mu g^{-1}$ DNA) compared with CON. Serum from offspring of restricted- and over-fed ewes had no effect on basal respiration ($P \geq 0.32$; CON = 34.32 ± 3.88, RES = 44.87 ± 3.88, OVER = 35.38 ± 3.88, pmol $O_2 \cdot min^{-1} \cdot \mu g^{-1}$ DNA), ATP production ($P \geq 0.69$; CON = 19.33 ± 3.21, RES = 22.54 ± 3.21, OVER = 18.94 ± 3.21 pmol $O_2 \cdot min^{-1} \cdot \mu g^{-1}$ DNA), maximal respiration ($P \geq 0.17$; CON = 177.15 ± 49.83, RES = 303.21 ± 49.83, OVER = 182.58 ± 49.83 pmol $O_2 \cdot min^{-1} \cdot \mu g^{-1}$ DNA), spare respiratory capacity ($P \geq 0.19$; CON = 142.83 ± 47.79, RES = 258.34 ± 47.79, OVER = 147.19 ± 47.79 pmol $O_2 \cdot min^{-1} \cdot \mu g^{-1}$ DNA), or non-mitochondrial respiration ($P \geq 0.25$; CON = 11.75 ± 5.42, RES = 25.22 ± 5.42, OVER = 16.77 ± 5.42 pmol $O_2 \cdot min^{-1} \cdot \mu g^{-1}$ DNA) compared with CON. There was no effect of gender ($P \geq 0.22$) for any of the variables analyzed for mitochondrial function.

Serum from offspring of restricted- and over-fed ewes had no effect on C2C12 glycolytic function (Figure 4). The variables of glycolysis ($P \geq 0.27$; CON = 48.03 ± 4.08, RES = 45.21 ±

4.08, OVER = 41.39 ± 4.08 mPh ·min^{-1} ·µg^{-1} DNA), glycolytic capacity ($P \geq 0.51$; CON = 51.41 ± 4.75, RES = 52.35 ± 4.75, OVER = 47.85 ± 4.75 mPh ·min^{-1} ·µg^{-1} DNA), glycolytic reserve ($P \geq 0.19$; CON = 3.38 ± 1.91, RES = 7.14 ± 1.91, OVER = 6.46 ± 1.91 mPh ·min^{-1} ·µg^{-1} DNA), and non-glycolytic acidification ($P \geq 0.40$; CON = 15.63 ± 1.93, RES = 16.69 ± 1.93, OVER = 14.33 ± 1.93 mPh ·min^{-1} ·µg^{-1} DNA) were not affected by treatment. There was no effect of gender ($P \geq 0.28$) for any of the variables analyzed for glycolytic function.

Muscle Metabolome Analysis

A total of 612 metabolites were detected in LM samples. Maternal diet altered metabolite concentrations at d 90, d 135, and birth (Table 1). At d 90, 86 metabolites were altered in RES offspring ($P \leq 0.05$), 19 increased and 67 decreased, compared with CON. At d 135, 72 metabolites were altered in RES offspring ($P \leq 0.05$), 22 increased and 50 decreased, compared with CON. At birth, 64 metabolites were altered RES offspring ($P \leq 0.05$), 14 increased and 50 decreased, compared with CON. The OVER offspring had a total of 78 metabolites altered at d 90 compared with CON ($P \leq 0.05$), with 49 metabolites increased and 29 decreased. At d 135 OVER offspring had 27 metabolites altered ($P \leq 0.05$), 18 increased and 9 decreased, compared with CON. At birth, OVER offspring had 102 metabolites altered ($P \leq 0.05$), 15 increased and 87 decreased, compared with CON. At d 90 RES offspring had 104 metabolites altered ($P \leq 0.05$), 20 increased and 84 decreased, compared with OVER. At d 135, RES offspring had 118 metabolites altered ($P \leq 0.05$), 38 increased and 80 decreased, compared with OVER. At birth, RES offspring had 117 metabolites altered ($P \leq 0.05$), 85 increased and 32 decreased, compared with OVER.

Day of gestation altered metabolite concentrations within each treatment group (Table 2). In the CON treatment group, 362 metabolites were altered at d 90 compared with d 135, with 200 increased and 162 decreased. A total of 436 metabolites were altered at d 90 compared with birth, with 191 increased and 245 decreased. Compared with birth, 374 metabolites were altered at d 135, with 127 increased and 247 decreased. In the RES treatment group, 376 metabolites were altered at d 90 compared with d 135, with 211 increased and 165 decreased. Compared with birth, 441 metabolites were altered at d 90, with 189 increased and 252 decreased. Additionally, 374 metabolites were altered at d 135 compared with birth, with 129 increased and 245 decreased. In the OVER treatment group, 374 metabolites were altered at d 90 compared with d 135, with 219 increased and 155 decreased. A total of 441 metabolites were altered at d 90 compared with birth, with 230 increased and 211 decreased. There were 365 metabolites altered at d 135 compared with birth, with 160 increased and 205 decreased. Principle component analysis of LM samples (Figure 5) demonstrated close clustering in response to day of gestation, indicating that time is the primary driver of variance between samples.

Both maternal over- and restricted-feeding altered metabolites in each of the 8 major metabolic pathways (Table 3). For amino acid metabolism, and 63 of 161 identified metabolites were altered in OVER compared with CON ($P < 0.05$). For peptide metabolism, 6 of 17 identified metabolites were altered in OVER compared with CON ($P < 0.05$). For carbohydrate metabolism, 12 of 39 identified metabolites were altered in OVER compared with CON ($P < 0.05$). For energy metabolism, 1 of 11 identified metabolites were altered in OVER compared with CON ($P < 0.05$). For lipid metabolism 70 of 268 identified metabolites were altered in OVER compared with CON ($P < 0.05$). For nucleotide metabolism 10 of 56 identified metabolites were altered in OVER compared with CON ($P < 0.05$). For cofactors and vitamins,

15 of 31 identified metabolites were altered in OVER compared with CON ($P < 0.05$). For xenobiotics, 10 of 29 identified metabolites were altered in OVER compared with CON ($P < 0.05$). Maternal restricted-feeding additionally altered metabolites in each of the 8 major metabolic pathways. For amino acid metabolism, 53 of 161 metabolites were altered in RES compared with CON ($P < 0.05$). For peptide metabolism, 4 of 17 metabolites were altered in RES compared with CON ($P < 0.05$). For carbohydrate metabolism, 8 of 39 metabolites were altered in RES compared with CON ($P < 0.05$). For energy metabolism, 1 of 11 metabolites were altered in RES compared with CON ($P < 0.05$). For lipid metabolism 89 of 268 metabolites were altered in RES compared with CON ($P < 0.05$). For Nucleotide metabolism 16 of 56 metabolites were altered in RES compared with CON ($P < 0.05$). For cofactors and vitamins, 12 of 31 metabolites were altered in RES compared with CON ($P < 0.05$). For xenobiotics, 13 of 29 metabolites were altered in RES compared with CON ($P < 0.05$).

A total of 43 pathways were altered by the interaction of treatment and time, including fatty acid metabolism, lysophospholipid metabolism, and sphingolipid metabolism (Table 4). Maternal diet altered 51 pathways, including phosphatidylethanolamine metabolism, histidine metabolism, and phosphatidylcholine metabolism (Table 5). Day of gestation altered 85 pathways including ceramide metabolism, diacylglycerol metabolism, acyl carnitine metabolism, and branched chain amino acid metabolism (Table 6). In RES offspring, 33 pathways were altered at d 90 (Table 7, 39 pathways were altered at d 135 (Table 8), and 32 pathways were altered at birth (Table 9) compared with CON. In OVER offspring, 40 pathways were altered at d 90 (Table 10), 19 pathways were altered at d 135 (Table 11), and 43 pathways were altered at birth (Table 12) compared with CON.

Maternal over-feeding alters carbohydrate metabolism in the offspring

Maternal over-feeding altered N6-carboxymethyllysine, an advanced glycation end product, in the offspring (Figure 6). No changes were observed between OVER and CON at d 90 or 135, but at birth, N6-carboxymethyllysine was increased 1.61-fold compared with CON (P = 0.02).

Maternal over-feeding alters amino acid metabolism in the offspring

Increased maternal nutrient consumption altered alanine and aspartate metabolism in the offspring (Figure 7). At d 90, alanine was increased (fold change = 1.21; P = 0.03) in OVER compared with CON. However, N-acetylasparagine (fold change = 0.56; P = 0.001) and hydroxyasparagine (fold change = 0.75; P = 0.04) were decreased in OVER compared with CON. At d 135 N-acetylaspartate (NAA; fold change = 0.63; P = 0.03) was decreased. At birth N-methylalanine (fold change = 1.32; P = 0.04) was increased, and alanine (fold change = 0.80; P = 0.01), N-acetylalanine (fold change = 0.71; P = 0.001), aspartate (fold change = 0.60; P = 0.002), asparagine (fold change = 0.66; P = 0.02), and hydroxyasparagine (fold change = 0.72; P = 0.03) were decreased in OVER compared with CON.

The OVER offspring also exhibited changes to glutathione metabolism (Figure 8). At d 90, reduced glutathione (GSH; fold change = 2.62; P = 0.005), was increased in OVER compared with CON, while cystine-glutathione disulfide (fold change = 0.36; P = 0.04) and 2-aminobutyrate (fold change = 0.54; P = 0.03) were increased. At d 135, opthalmate (fold change = 0.38; P = 0.0002) was decreased, and at birth S-methylglutathione (fold change = 0.61; P = 0.04) was decreased in OVER compared with CON.

Increased maternal nutrient consumption impacted histidine metabolism (Figure 9). At d 90, maternal over-feeding increased carnosine (fold change = 1.18; $P = 0.03$) and decreased 1-methylhistidine (fold change = 0.57; $P = 0.007$), 3-methylhistidine (fold change = 0.60; $P = 0.007$), and N-acetyl-1-methylhistidine (fold change = 0.25; $P = 0.00001$) compared with CON. At d 135, OVER offspring exhibited increased imidazole propionate (fold change = 2.73; $P = 0.03$) and formiminoglutamate (fold change = 2.27; $P = 0.05$) compared with CON. At birth, 1-methylhistamine was decreased 0.50-fold ($P = 0.03$) in OVER compared with CON.

Maternal over-feeding resulted in changes to leucine, isoleucine and valine metabolism in the offspring muscle (Figure 10). 4-methyl-2-oxopentanoate (fold change = 0.56; $P = 0.04$) was decreased, whereas beta-hydroxyisovalerate (fold change = 2.09; $P = 0.002$), beta-hydroxyisovaleroylcarnitine (fold change = 1.56; $P = 0.01$), 3-methylglutaconate (fold change = 1.89; $P = 0.01$), and 3-hydroxyisobutyrate (fold change = 1.58; $P = 0.02$) were increased at d 90 in OVER compared with CON. At d 135, beta-hydroxyisovalerate (fold change = 1.58; $P = 0.02$), 3-methylglutaconate (fold change = 1.76; $P = 0.01$), and 3-hydroxyisobutyrate (fold change = 1.59; $P = 0.01$) continued to be increased.

The OVER offspring exhibited divergent tryptophan metabolism (Figure 11). N-formylanthranilic acid (fold change = 2.16; $P = 0.01$) and indolelactate (fold change = 1.68; $P = 0.007$) were increased while 3-indoxyl sulfate (fold change = 0.59; $P = 0.04$) was decreased at d 90 in OVER compared with CON. Indolelactate (fold change = 1.58; $P = 0.01$) was increased at d 135 in OVER offspring. A birth, indoleacetate (fold change = 0.49; $P = 0.05$) and 3-indoxyl sulfate (fold change = 0.36; $P = 0.0003$) were decreased in offspring from over-fed ewes when compared with CON.

Finally, maternal over-feeding caused changes to tyrosine metabolism in the offspring (Figure 12). Tyrosine (fold change = 1.41; $P = 0.02$), 3-(4-hydroxyphenyl)lactate (fold change = 1.90; $P = 0.001$), and phenol sulfate (fold change = 1.44; $P = 0.04$) were increased at d 90 in OVER compared with CON, while 3-methoxytyrosine (fold change = 0.49; $P = 0.01$) was decreased. 3-methoxytyrosine (fold change = 0.60; $P = 0.03$) continued to be decreased in OVER at d 135. There were no changes observed at birth.

Maternal over-feeding alters offspring lipid metabolism

Over-feeding altered fatty acid and branched chain amino acid metabolism at birth by decreasing propionylcarnitine (C3) 0.35-fold ($P = 0.01$; Figure 13). Maternal over-feeding caused no changes to this pathway at d 90 and 135. Offspring of over-fed ewes exhibited changes to fatty acid and acyl carnitine metabolism (Figure 14). At d 90 and 135, no metabolites involved in fatty acid and acyl carnitine metabolism were altered compared with CON. However, at birth, 3-hydroxybutyrylcarnitine was increased 1.76-fold ($P = 0.04$). At the same time-point, OVER exhibited decreased 5-dodecenoylcarnitine (C12:1; fold change = 0.55; $P = 0.003$), linolenoylcarnitine (C18:3; fold change = 0.78; $P = 0.006$), myristoleoylcarnitine (C14:1; fold change = 0.60; $P = 0.02$), adrenoylcarnitine (C22:4; fold change = 0.45; $P = 0.001$), behenoylcarnitine (C22; fold change = 0.46; $P = 0.01$), docosatrienoylcarnitine (C22:3; fold change = 0.54; $P = 0.01$), docosapentaenoylcarnitine (C22:5n3; fold change = 0.44; $P = 0.0009$), docosahexaenoylcarnitine (C22:6; fold change = 0.61; $P = 0.005$), margaroylcarnitine (C17; fold change = 0.67; $P = 0.03$), and nervonoylcarnitine (C24:1) (fold change = 0.34; $P = 0.003$) compared with CON.

Lysophospholipid metabolism was altered by maternal over-feeding, especially at birth (Figure 15). At d 90, 1-palmitoleoyl-GPC (16:1; fold change = 1.22; *P* = 0.03) and 1-oleoyl-GPG (18:1; fold change = 1.39; *P* = 0.02) were increased in OVER compared with CON. No changes were identified at d 135. However, at birth, 13 lysophospholipid metabolites were decreased compared with CON [1-palmitoyl-GPC (16:0; fold change = 0.82; *P* = 0.03), 1-palmitoleoyl-GPC (16:1; fold change = 0.82; *P* = 0.04), 1-stearoyl-GPC (18:0; fold change = 0.75; *P* = 0.005), 1-oleoyl-GPC (18:1; fold change = 0.76; *P* = 0.0008), 1-linoleoyl-GPC (18:2; fold change = 0.54; *P* = 0.005), 1-palmitoyl-GPE (16:0; fold change = 0.63; *P* = 0.0001), 1-stearoyl-GPE (18:0; fold change = 0.71; *P* = 0.0003), 1-oleoyl-GPE (18:1; fold change = 0.75; *P* = 0.0006), 1-linoleoyl-GPE (18:2; fold change 0.51= ; *P* = 0.0008), 1-arachidonoyl-GPE (20:4n6; fold change = 0.65; *P* = 0.02), 1-palmitoyl-GPI (16:0; fold change = 0.68; *P* = 0.03), 1-stearoyl-GPI (18:0; fold change = 0.63; *P* = 0.02), and 1-oleoyl-GPI (18:1; fold change = 0.61; *P* = 0.02)].

Maternal over-feeding also caused extensive changes to metabolites involved phosphatidylethanolamine metabolism, especially at the birth time-point (Figure 16). No changes were identified at d 90. At d 135, 1,2-dipalmitoyl-GPE (16:0/16:0; fold change = 1.44; P = 0.01) was increased at in OVER compared with CON. At birth, 10 of 11 metabolites identified in this pathway were decreased in OVER compared with CON [1-palmitoyl-2-oleoyl-GPE (16:0/18:1; fold change = 0.68; P = 0.002), 1-palmitoyl-2-arachidonoyl-GPE (16:0/20:4; fold change = 0.61; P = 0.002), 1-palmitoyl-2-docosahexaenoyl-GPE (16:0/22:6; fold change = 0.54; P = 0.002), 1-palmitoleoyl-2-oleoyl-GPE (16:1/18:1; fold change = 0.68; P = 0.006), 1-stearoyl-2-oleoyl-GPE (18:0/18:1; fold change = 0.67; P = 0.0), 1-stearoyl-2-arachidonoyl-GPE (18:0/20:4; fold change = 0.70; P = 0.0), 1,2-dioleoyl-GPE (18:1/18:1; fold change = 0.67; P = 0.005), 1-oleoyl-2-linoleoyl-GPE (18:1/18:2; fold change = 0.47; P = 0.01), 1-oleoyl-2-arachidonoyl-GPE

(18:1/20:4; fold change = 0.73; P = 0.006), and 1-oleoyl-2-docosahexaenoyl-GPE (18:1/22:6; fold change = 0.73; P = 0.03)].

Maternal restricted-feeding alters offspring amino acid metabolism

Lambs born to restricted-fed ewes exhibited changes in muscle glutamate metabolism (Figure 17). Glutamate, gamma-methyl ester (fold change = 1.53; *P* = 0.006) and gamma-aminobutyrate (GABA; fold change = 1.27; *P* = 0.03) were increased at d 90 in RES compared with CON. Glutamate (fold change = 0.85; *P* = 0.03) was decreased at d 135 and remained decreased at birth (fold change = 0.78; *P* = 0.0006) along with N-methyl-GABA (fold change = 0.64; *P* = 0.006) in RES compared with CON.

Glutathione metabolism was also impacted by restricted-maternal diet (Figure 18). At d 90, reduced glutathione (GSH) was increased 3.07-fold (*P* = 0.008), and 2-aminobutyrate was decreased 0.54-fold (*P* = 0.005) in RES compared with CON. At d 135, oxidized glutathione (GSSG) was decreased 0.21-fold (*P* = 0.02) and 2-aminobutyrate (*P* = 0.001) was increased 1.93-fold in RES compared with CON. There were no changes to glutathione metabolism at birth in offspring from restricted-fed mothers.

Restricted-feeding altered offspring muscle histidine metabolism (Figure 19). No changes were observed at d 90. However, at d 135, 1-methylhistidine (fold change = 2.69; *P* = 0.00001), 3-methylhistidine (fold change = 3.38; *P* = 0.00001), N-acetyl-1-methylhistidine (fold change = 3.74; *P* = 0.0), formiminoglutamate (fold change = 2.95; *P* = 0.00001), and anserine (fold change = 1.41; *P* = 0.003) were increased while imidazole propionate (fold change = 0.20; *P* = 0.008) and carnosine (fold change = 0.85; *P* = 0.03) were decreased in RES compared with CON. Additionally, at birth, imidazole lactate (fold change = 0.64; *P* = 0.05), carnosine (fold change =

0.80; *P* = 0.003), anserine (fold change = 0.80; *P* = 0.03), and 1-methyl-4-imidazoleacetate (fold change = 0.63; *P* = 0.03) were all decreased in RES compared with CON.

Maternal restricted-feeding alters offspring lipid metabolism

Maternal restricted-nutrition caused reductions to offspring lysophosphlipids at all time points (Figure 20). At d 90, 1-palmitoyl-GPE (16:0; fold change = 0.81; *P* = 0.04) and 1-stearoyl-GPE (18:0; fold change = 0.83; *P* = 0.05) were decreased in RES compared with CON. At d 135, 1-stearoyl-GPE (18:0; fold change = 0.68; *P* = 0.001), 1-stearoyl-GPE (18:0; fold change = 0.51; *P* = 0.00001), and 1-stearoyl-GPI (18:0; fold change = 0.58; *P* = 0.0003) were decreased in RES compared with CON. At birth, 1-palmitoyl-GPC (16:0; fold change = 0.75; *P* = 0.002), 1-palmitoleoyl-GPC (16:1; fold change = 0.72; *P* = 0.0007), 1-stearoyl-GPC (18:0; fold change = 0.81; *P* = 0.02), 1-oleoyl-GPC (18:1; fold change = 0.83; *P* = 0.009), 1-palmitoyl-GPE (16:0; fold change = 0.80; *P* = 0.05), 1-stearoyl-GPE (18:0; fold change = 0.70; *P* = 0.0001), and 1-oleoyl-GPE (18:1; fold change = 0.84; *P* = 0.02) were all decreased in RES compared with CON.

Restricted-feeding cause changes in offspring phosphatidylcholine metabolism at all time points (Figure 21). 1-palmitoyl-2-oleoyl-GPC (16:0/18:1; fold change = 0.84; *P* = 0.005), 1-palmitoyl-2-linoleoyl-GPC (16:0/18:2; fold change = 0.84; *P* = 0.01), 1-palmitoyl-2-dihomo-linolenoyl-GPC (16:0/20:3n3 or 6; fold change = 0.66; *P* = 0.0002), 1-stearoyl-2-oleoyl-GPC (18:0/18:1; fold change = 0.75; *P* = 0.004), and 1,2-dioleoyl-GPC (18:1/18:1; fold change = 0.86; *P* = 0.008) were all reduced at d 90 in RES compared with CON. At d 135, 1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1; fold change = 0.84; *P* = 0.04) was decreased and 1-palmitoleoyl-2-linolenoyl-GPC (16:1/18:3; fold change = 1.39; *P* = 0.04) was increased in RES compared

with CON. Additionally, at birth, 1-myristoyl-2-palmitoyl-GPC (14:0/16:0; fold change = 0.81; *P* = 0.03), 1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1; fold change = 0.76; *P* = 0.002), 1-palmitoyl-2-oleoyl-GPC (16:0/18:1; fold change = 0.87; *P* = 0.03), and 1-palmitoyl-2-dihomo-linolenoyl-GPC (16:0/20:3n3 or 6; fold change = 0.81; *P* = 0.04) were all decreased in RES compared with CON.

There were also changes in phosphatidylethanolamine metabolism in response to maternal restricted-nutrition (Figure 22). At d 90, 1-palmitoyl-2-oleoyl-GPE (16:0/18:1; fold change = 0.73; *P* = 0.007), 1-palmitoleoyl-2-oleoyl-GPE (16:1/18:1; fold change = 0.72; *P* = 0.02), 1-stearoyl-2-oleoyl-GPE (18:0/18:1; fold change = 0.70; *P* = 0.007), and 1,2-dioleoyl-GPE (18:1/18:1; fold change = 0.77; *P* = 0.01) were reduced in RES compared with CON. Despite seeing no changes to any metabolites at d 135, 1-palmitoleoyl-2-oleoyl-GPE (16:1/18:1; fold change = 0.75; *P* = 0.03) was again reduced at birth in RES compared with CON.

Finally, restricted-feeding resulted in broad changes to offspring sphingolipid metabolism, especially at d 90 (Figure 23). Myristoyl dihydrosphingomyelin (d18:0/14:0; fold change = 0.71; *P* = 0.01), palmitoyl dihydrosphingomyelin (d18:0/16:0; fold change = 0.65; *P* = 0.0004), palmitoyl sphingomyelin (d18:1/16:0; fold change = 0.84; *P* = 0.005), stearoyl sphingomyelin (d18:1/18:0; fold change = 0.82; *P* = 0.008), behenoyl sphingomyelin (d18:1/22:0; fold change = 0.56; *P* = 0.0002), tricosanoyl sphingomyelin (d18:1/23:0; fold change = 0.50; *P* = 0.0004), lignoceroyl sphingomyelin (d18:1/24:0; fold change = 0.55; *P* = 0.0007), sphingomyelin (d18:1/17:0, d17:1/18:0, d19:1/16:0; fold change = 0.75; *P* = 0.009), sphingomyelin (d18:1/20:0, d16:1/22:0; fold change = 0.65; *P* = 0.003), sphingomyelin (d18:1/20:1, d18:2/20:0; fold change = 0.73; *P* = 0.007), sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1; fold change = 0.68; *P* = 0.002), sphingomyelin (d18:2/23:0, d18:1/23:1, d17:1/24:1;

fold change = 0.68; *P* = 0.03), sphingomyelin (d18:1/24:1, d18:2/24:0; fold change = 0.64; *P* = 0.006), sphingomyelin (d18:2/24:1, d18:1/24:2; fold change = 0.77; *P* = 0.03), sphingomyelin (d18:0/18:0, d19:0/17:0; fold change = 0.58; *P* = 0.001), and sphingomyelin (d18:1/19:0, d19:1/18:0; fold change = 0.57; *P* = 0.02) were decreased at d 90 in RES compared with CON. At d 135, sphingomyelin (d18:2/14:0, d18:1/14:1; fold change = 1.24; *P* = 0.02) and sphingomyelin (d18:2/24:2; fold change = 2.02; *P* = 0.002) were increased, whereas at birth, sphingomyelin (d18:1/20:1, d18:2/20:0; fold change = 0.80; *P* = 0.007) and sphingomyelin (d18:1/19:0, d19:1/18:0; fold change = 0.61; *P* = 0.04) were decreased in RES compared with CON.

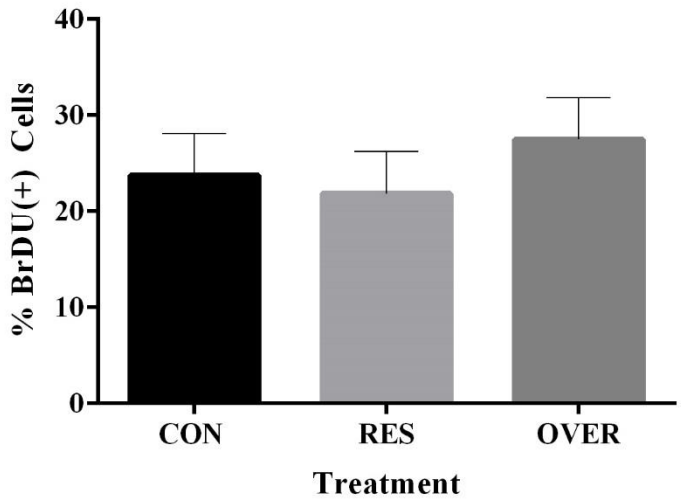
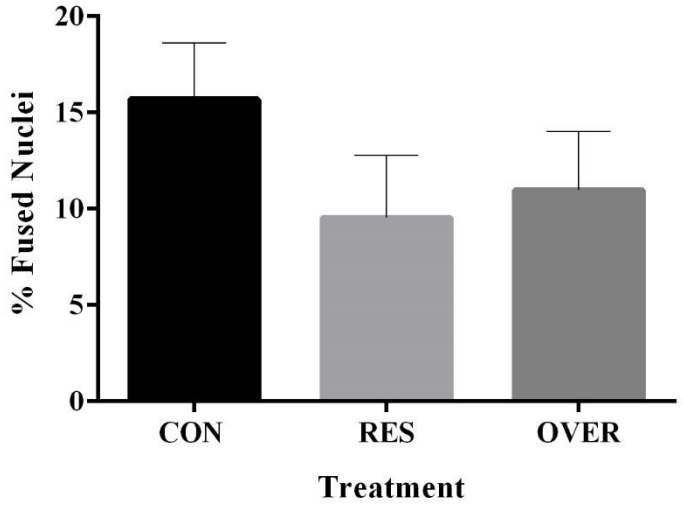


Figure 1: Serum from offspring of poorly nourished ewes does not alter C2C12 proliferation. C2C12 cells were cultured in serum collected from offspring of CON (n = 6), RES (n = 6), or OVER (n = 6) ewes at birth. Percentage of cells in s-phase was measured by 5-bromo-2'-deoxyuridine (BrdU) incorporation in C2C12 cells cultured in offspring serum for 48 hours. Cells were cultured in duplicate wells, and the experiment was replicated once. Data are presented as mean ± standard error.

A



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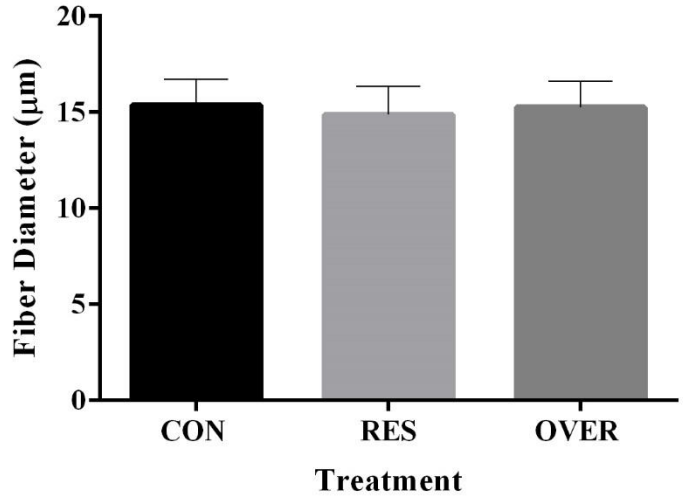
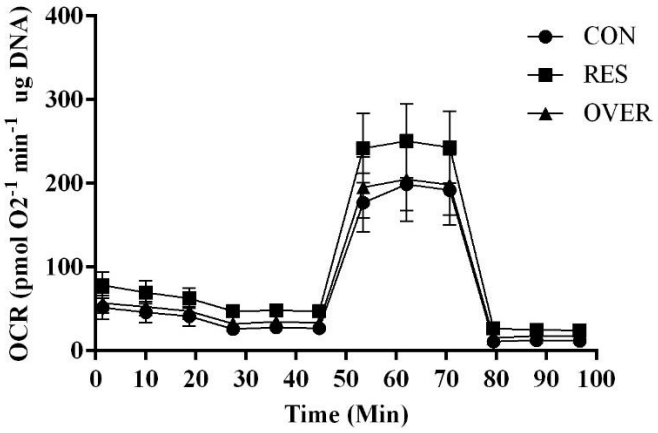


Figure 2: Serum from offspring of poorly nourished ewes does not alter C2C12 differentiation. C2C12 cells were cultured in serum collected from offspring of CON (n = 6), RES (n = 6), or OVER (n = 6) ewes at birth. The fusion index (A) and myofiber diameter (B) of differentiated C2C12 myofibers cultured in offspring serum for 5 days were calculated. Cells were cultured in duplicate wells, and the experiment was replicated once. Data are presented as mean ± standard error.

A



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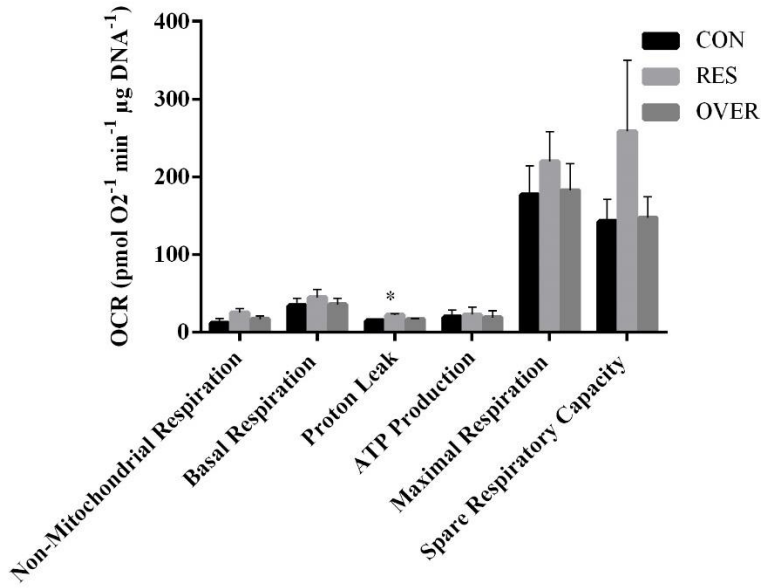

Figure 3: Serum from offspring born to restricted-fed ewes alters C2C12 mitochondrial respiration. C2C12 cells were cultured in serum collected from offspring of CON (n = 6), RES (n = 6), or OVER (n = 6) ewes at birth. (A) Oxygen Consumption Rate (OCR), an indicator of oxygen-dependent mitochondrial ATP production, was measured when C2C12 cells were successively exposed to oligomycin, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and rotenone/antimycin. (B) Calculation of non-mitochondrial respiration, basal respiration, proton leak, ATP production, maximal respiration, and spare respiratory capacity of C2C12. Data are presented as mean ± standard error. * denotes $P \leq 0.05$ compared with CON.

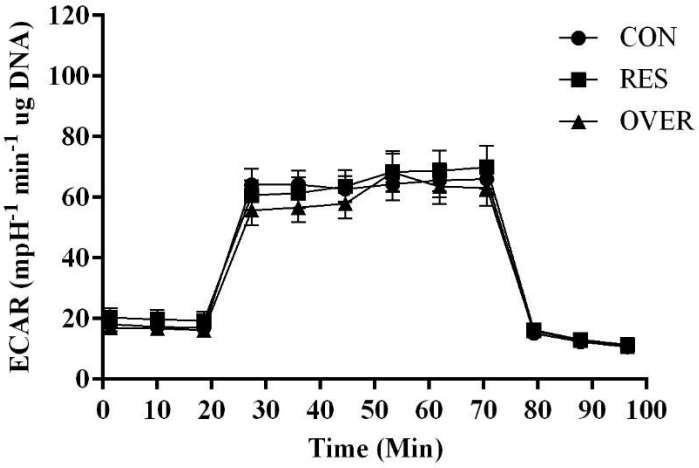

Figure 4: Serum from offspring of poorly nourished ewes does not alter C2C12 glycolytic function. C2C12 cells were cultured in serum collected from offspring of control-fed (CON, n = 6), restricted (RES, n = 6), or over-fed (OVER, n = 6) ewes at birth. Extracellular Acidification Rate (ECAR), an indication of glycolysis, was measured when C2C12 cells were sequentially exposed to glucose, oligomycin, and 2-deoxy-D-glucose (2-DG).

Table 1. Number of fetal metabolites in LD altered by maternal diet.

		Metabolites $P \leq 0.05$			Metabolites $0.05 < P \leq 0.1$		
Diet	Time	Total	Increased	Decreased	Total	Increased	Decreased
RES/CON	d 90	86	19	67	35	9	26
	d 135	72	22	50	28	6	22
	Birth	64	14	50	41	12	29
OVER/CON	d 90	78	49	29	30	20	10
	d 135	27	18	9	22	13	9
	Birth	102	15	87	50	9	41
RES/OVER	d 90	104	20	84	39	9	30
	d 135	118	38	80	38	13	25
	Birth	117	85	32	56	44	12

[1] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

Table 2. Number of fetal metabolites in LD altered by day of gestation.

			Metabolites $P \leq 0.05$			Metabolites $0.05 < P \leq 0.1$	
Diet	Time	Total	Increased	Decreased	Total	Increased	Decreased
CON	d 90/d 135	362	200	162	28	15	13
	d 90/Birth	436	191	245	29	13	16
	d 135/Birth	374	127	247	35	17	18
RES	d 90/d 135	376	211	165	31	15	16
	d 90/Birth	441	189	252	32	15	17
	d 135/Birth	374	129	245	33	17	16
OVER	d 90/d 135	374	219	155	51	38	13
	d 90/Birth	441	230	211	20	11	9
	d 135/Birth	365	160	205	42	16	26

[1] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

Table 3. Number of metabolites in major pathways altered by treatment.

Major Metabolic Pathway	Total Number of Metabolites in Pathway	Pathway Metabolites Altered in OVER vs. CON	Pathway Metabolites Altered in RES vs. CON
Amino Acid	161	63	53
Peptide	17	6	4
Carbohydrate	39	12	8
Energy	11	1	1
Lipid	268	70	89
Nucleotide	56	10	16
Cofactors and Vitamins	31	15	12
Xenobiotics	29	10	13

[1] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

[2]Significance is determined at $P \leq 0.05$ compared with CON. Total number of metabolites is determined from combining d 90, d 135, and birth time-points.

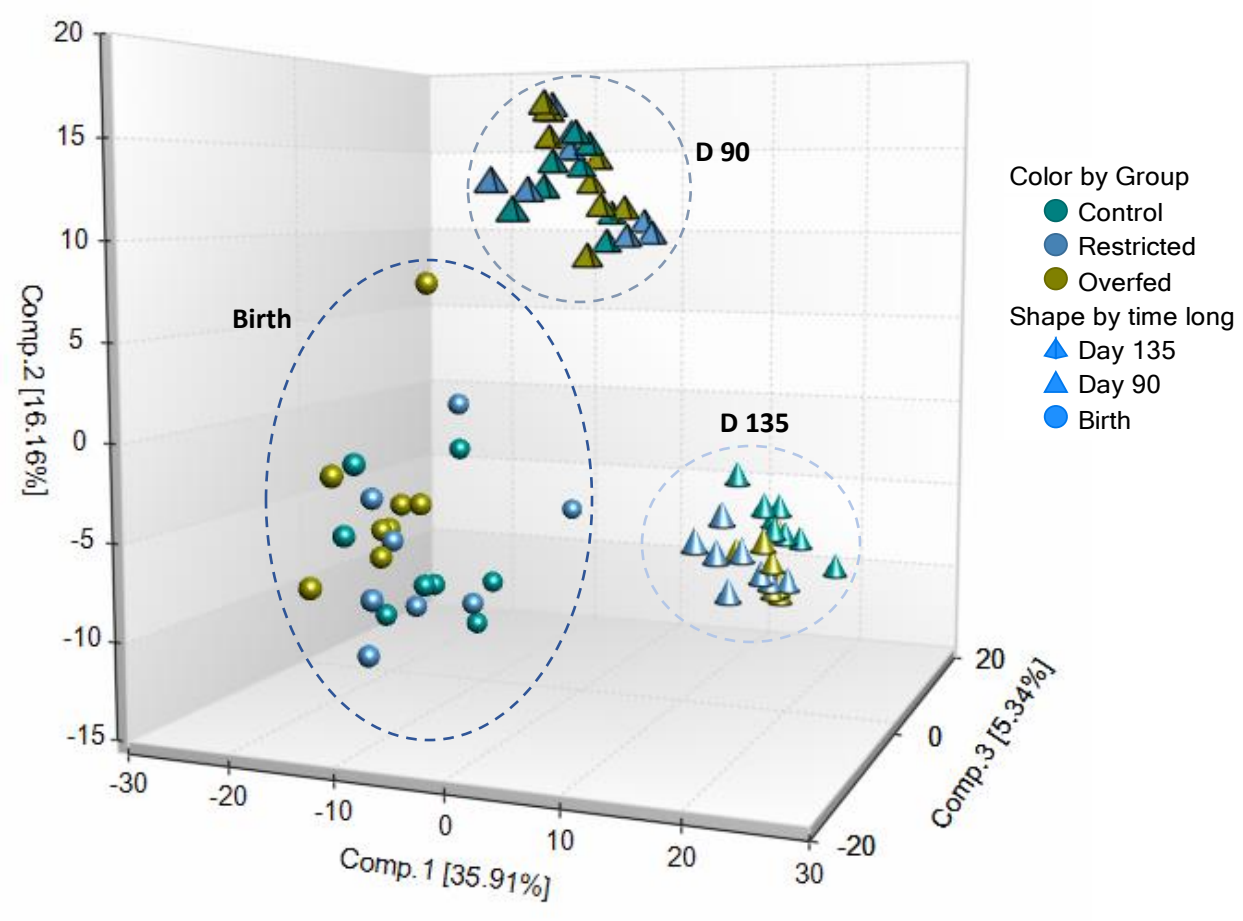

Figure 5: Principle Component Analysis (PCA) and hierarchical clustering obtained from employing metabolic concentration of all metabolites detected in LD tissue. PCA of LD samples indicates that time is the primary driver of variance between samples. LD samples (n = 8 per treatment per time point) were collected from offspring born to ewes fed CON, RES, or OVER diets at d 90, 135 of gestation, or within 24 hours of birth were analyzed by UPLC-MS/MS.

Table 4: Pathways altered by treatment across time.

Pathway Name	Pathway Metabolites			Enrichment [1] (k/m)/(n/N)
	Significant (k)	Detected (m)	Significant/ Detected (k/m)	
Acetylated Peptides	1	1	1	5.37
Bacterial/Fungal	1	1	1	5.37
Ketone Bodies	1	1	1	5.37
Riboflavin Metabolism	2	2	1	5.37
Tocopherol Metabolism	1	1	1	5.37
Vitamin A Metabolism	1	1	1	5.37
Medium Chain Fatty Acid	3	5	0.6	3.22
Fatty Acid Metabolism (also BCAA Metabolism)	1	2	0.5	2.68
Pentose Phosphate Pathway	1	2	0.5	2.68
Primary Bile Acid Metabolism	3	6	0.5	2.68
Pyrimidine Metabolism, Thymine containing	1	2	0.5	2.68
Vitamin B6 Metabolism	1	2	0.5	2.68
Sphingolipid Metabolism	15	31	0.48	2.6
Lysophospholipid	11	24	0.46	2.46
Phosphatidylethanolamine	5	11	0.45	2.44
Histidine Metabolism	6	14	0.43	2.3
Chemical	2	5	0.4	2.15
Hemoglobin and Porphyrin Metabolism	2	5	0.4	2.15
Nucleotide Sugar	2	5	0.4	2.15
Urea cycle; Arginine and Proline Metabolism	6	16	0.38	2.01
Pantothenate and CoA Metabolism	1	3	0.33	1.79
Benzoate Metabolism	3	10	0.3	1.61
Glutathione Metabolism	3	10	0.3	1.61
Nicotinate and Nicotinamide Metabolism	3	10	0.3	1.61
Plasmalogen	3	10	0.3	1.61
Methionine, Cysteine, SAM and Taurine Metabolism	5	17	0.29	1.58
Glycine, Serine and Threonine Metabolism	3	11	0.27	1.46

Alanine and Aspartate Metabolism	2	8	0.25	1.34
Food Component/Plant	3	13	0.23	1.24
Gamma-glutamyl Amino Acid	3	13	0.23	1.24
Glutamate Metabolism	3	13	0.23	1.24
Fatty Acid, Dicarboxylate	2	9	0.22	1.19
Lysoplasmalogen	1	5	0.2	1.07
Phosphatidylcholine	3	17	0.18	0.95
Lysine Metabolism	2	12	0.17	0.89
Fructose, Mannose and Galactose Metabolism	1	7	0.14	0.77
Tyrosine Metabolism	1	7	0.14	0.77
Purine Metabolism, (Hypo) Xanthine/Inosine containing	1	11	0.09	0.49
Tryptophan Metabolism	1	11	0.09	0.49
Leucine, Isoleucine and Valine Metabolism	2	24	0.08	0.45
Pyrimidine Metabolism, Uracil containing	1	12	0.08	0.45
Fatty Acid Metabolism (Acyl Carnitine)	1	34	0.03	0.16

[1] All compounds; Significant (n) = 114, Detected (N) = 612, Significant/ detected (n/N) = 0.19

[2] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

Table 5: Pathways altered by treatment.

Pathway Name	Pathway Metabolites			
	Significant (k)	Detected (m)	Significant/ Detected (k/m)	Enrichment [1] (k/m)/(n/N)
Acetylated Peptides	1	1	1	4.6
Advanced Glycation End-product	1	1	1	4.6
Bacterial/Fungal	1	1	1	4.6
Phosphatidylglycerol	1	1	1	4.6
Thiamine Metabolism	2	2	1	4.6
Phosphatidylethanolamine	7	11	0.64	2.93
Carnitine Metabolism	1	2	0.5	2.3
Guanidino and Acetamido Metabolism	1	2	0.5	2.3
Histidine Metabolism	7	14	0.5	2.3
Primary Bile Acid Metabolism	3	6	0.5	2.3
Pyrimidine Metabolism, Orotate containing	1	2	0.5	2.3
Secondary Bile Acid Metabolism	2	4	0.5	2.3
Methionine, Cysteine, SAM and Taurine Metabolism	8	17	0.47	2.17
Phosphatidylcholine	8	17	0.47	2.17
Food Component/Plant	6	13	0.46	2.12
Glutamate Metabolism	6	13	0.46	2.12
Aminosugar Metabolism	3	7	0.43	1.97
Tyrosine Metabolism	3	7	0.43	1.97
Lysophospholipid	10	24	0.42	1.92
Hemoglobin and Porphyrin Metabolism	2	5	0.4	1.84
Lysoplasmalogen	2	5	0.4	1.84
Glycine, Serine and Threonine Metabolism	4	11	0.36	1.67
Dipeptide	1	3	0.33	1.53
Inositol Metabolism	1	3	0.33	1.53
Phenylalanine Metabolism	1	3	0.33	1.53
Benzoate Metabolism	3	10	0.3	1.38
Nicotinate and Nicotinamide Metabolism	3	10	0.3	1.38

Plasmalogen	3	10	0.3	1.38
Purine Metabolism, (Hypo) Xanthine/Inosine containing	3	11	0.27	1.25
Alanine and Aspartate Metabolism	2	8	0.25	1.15
Polyunsaturated Fatty Acid (n3 and n6)	3	12	0.25	1.15
Gamma-glutamyl Amino Acid	3	13	0.23	1.06
Polyamine Metabolism	2	9	0.22	1.02
Ascorbate and Aldarate Metabolism	1	5	0.2	0.92
Chemical	1	5	0.2	0.92
Endocannabinoid	1	5	0.2	0.92
Medium Chain Fatty Acid	1	5	0.2	0.92
Phospholipid Metabolism	2	10	0.2	0.92
Urea cycle; Arginine and Proline Metabolism	3	16	0.19	0.86
Tryptophan Metabolism	2	11	0.18	0.84
Leucine, Isoleucine and Valine Metabolism	4	24	0.17	0.77
Fructose, Mannose and Galactose Metabolism	1	7	0.14	0.66
Progestin Steroids	1	7	0.14	0.66
Fatty Acid, Dicarboxylate	1	9	0.11	0.51
Ceramides	1	10	0.1	0.46
Glutathione Metabolism	1	10	0.1	0.46
Pyrimidine Metabolism, Cytidine containing	1	10	0.1	0.46
Sphingolipid Metabolism	3	31	0.1	0.45
Purine Metabolism, Adenine containing	1	11	0.09	0.42
Lysine Metabolism	1	12	0.08	0.38
Fatty Acid Metabolism (Acyl Carnitine)	2	34	0.06	0.27

[1] All compounds; Significant (n) = 133, Detected (N) = 612, Significant/ detected (n/N) = 0.22

[2] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

Table 6: Pathways altered by time.

Pathway Name	Pathway Metabolites			Enrichment [1] (k/m)/(n/N)
	Significant (k)	Detected (m)	Significant/ Detected (k/m)	
Acetylated Peptides	1	1	1	1.07
Advanced Glycation End-product	1	1	1	1.07
Alanine and Aspartate Metabolism	8	8	1	1.07
Aminosugar Metabolism	7	7	1	1.07
Ascorbate and Aldarate Metabolism	5	5	1	1.07
Bacterial/Fungal	1	1	1	1.07
Carnitine Metabolism	2	2	1	1.07
Ceramides	10	10	1	1.07
Chemical	5	5	1	1.07
Corticosteroids	1	1	1	1.07
Creatine Metabolism	4	4	1	1.07
Diacylglycerol	9	9	1	1.07
Dipeptide	3	3	1	1.07
Endocannabinoid	5	5	1	1.07
Fatty Acid Metabolism	1	1	1	1.07
Fatty Acid Metabolism (Acyl Choline)	1	1	1	1.07
Fatty Acid Metabolism (also BCAA Metabolism)	2	2	1	1.07
Fatty Acid Metabolism (Acyl Carnitine)	34	34	1	1.07
Fatty Acid Metabolism (Acyl Glycine)	2	2	1	1.07
Fatty Acid Synthesis	2	2	1	1.07
Fatty Acid, Amino	1	1	1	1.07
Fatty Acid, Branched	2	2	1	1.07
Fatty Acid, Monohydroxy	5	5	1	1.07
Food Component/Plant	13	13	1	1.07
Gamma-glutamyl Amino Acid	13	13	1	1.07
Glutamate Metabolism	13	13	1	1.07
Glycerolipid Metabolism	3	3	1	1.07

Glycine, Serine and Threonine Metabolism	11	11	1	1.07
Guanidino and Acetamido Metabolism	2	2	1	1.07
Hemoglobin and Porphyrin Metabolism	5	5	1	1.07
Inositol Metabolism	3	3	1	1.07
Ketone Bodies	1	1	1	1.07
Medium Chain Fatty Acid	5	5	1	1.07
Mevalonate Metabolism	1	1	1	1.07
Monoacylglycerol	9	9	1	1.07
Nicotinate and Nicotinamide Metabolism	10	10	1	1.07
Pantothenate and CoA Metabolism	3	3	1	1.07
Phenylalanine Metabolism	3	3	1	1.07
Phosphatidylethanolamine	11	11	1	1.07
Phosphatidylglycerol	1	1	1	1.07
Phosphatidylinositol	2	2	1	1.07
Phosphatidylserine	3	3	1	1.07
Polyamine Metabolism	9	9	1	1.07
Polyunsaturated Fatty Acid (n3 and n6)	12	12	1	1.07
Pregnenolone Steroids	3	3	1	1.07
Progestin Steroids	7	7	1	1.07
Purine and Pyrimidine Metabolism	1	1	1	1.07
Purine Metabolism, Guanine containing	7	7	1	1.07
Pyrimidine Metabolism, Orotate containing	2	2	1	1.07
Pyrimidine Metabolism, Thymine containing	2	2	1	1.07
Pyrimidine Metabolism, Uracil containing	12	12	1	1.07
Riboflavin Metabolism	2	2	1	1.07
TCA Cycle	9	9	1	1.07
Thiamine Metabolism	2	2	1	1.07
Tocopherol Metabolism	1	1	1	1.07
Tyrosine Metabolism	7	7	1	1.07
Vitamin A Metabolism	1	1	1	1.07
Vitamin B6 Metabolism	2	2	1	1.07

Leucine, Isoleucine and Valine Metabolism	23	24	0.96	1.02
Lysophospholipid	23	24	0.96	1.02
Methionine, Cysteine, SAM and Taurine Metabolism	16	17	0.94	1
Phosphatidylcholine	16	17	0.94	1
Urea cycle; Arginine and Proline Metabolism	15	16	0.94	1
Histidine Metabolism	13	14	0.93	0.99
Lysine Metabolism	11	12	0.92	0.98
Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	10	11	0.91	0.97
Purine Metabolism, (Hypo) Xanthine/Inosine containing	10	11	0.91	0.97
Purine Metabolism, Adenine containing	10	11	0.91	0.97
Sphingolipid Metabolism	28	31	0.9	0.96
Benzoate Metabolism	9	10	0.9	0.96
Phospholipid Metabolism	9	10	0.9	0.96
Plasmalogen	9	10	0.9	0.96
Pyrimidine Metabolism, Cytidine containing	9	10	0.9	0.96
Fatty Acid, Dicarboxylate	8	9	0.89	0.95
Long Chain Fatty Acid	10	12	0.83	0.89
Pentose Metabolism	5	6	0.83	0.89
Glutathione Metabolism	8	10	0.8	0.85
Lysoplasmalogen	4	5	0.8	0.85
Nucleotide Sugar	4	5	0.8	0.85
Secondary Bile Acid Metabolism	3	4	0.75	0.8
Tryptophan Metabolism	8	11	0.73	0.78
Fructose, Mannose and Galactose Metabolism	5	7	0.71	0.76
Oxidative Phosphorylation	1	2	0.5	0.53
Pentose Phosphate Pathway	1	2	0.5	0.53
Primary Bile Acid Metabolism	3	6	0.5	0.53

[1] All compounds; Significant (n) = 574, Detected (N) = 612, Significant/ detected (n/N) = 0.94

[2] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was

performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

Table 7: Pathways altered at d 90 in RES compared with CON.

Pathway Name	Pathway Metabolites			
	Significant (k)	Detected (m)	Significant/ Detected (k/m)	Enrichment [1] (k/m)/(n/N)
Advanced Glycation End-product	1	1	1	7.12
Ceramides	7	10	0.7	4.98
Sphingolipid Metabolism	16	31	0.52	3.67
Plasmalogen	5	10	0.5	3.56
Thiamine Metabolism	1	2	0.5	3.56
Nucleotide Sugar	2	5	0.4	2.85
Food Component/Plant	5	13	0.38	2.74
Phosphatidylethanolamine	4	11	0.36	2.59
Inositol Metabolism	1	3	0.33	2.37
Pantothenate and CoA Metabolism	1	3	0.33	2.37
Primary Bile Acid Metabolism	2	6	0.33	2.37
Benzoate Metabolism	3	10	0.3	2.13
Nicotinate and Nicotinamide Metabolism	3	10	0.3	2.13
Pyrimidine Metabolism, Cytidine containing	3	10	0.3	2.13
Phosphatidylcholine	5	17	0.29	2.09
Glutathione Metabolism	2	10	0.2	1.42
Lysoplasmalogen	1	5	0.2	1.42
Medium Chain Fatty Acid	1	5	0.2	1.42
Pyrimidine Metabolism, Uracil containing	2	12	0.17	1.19
Glutamate Metabolism	2	13	0.15	1.09
Tyrosine Metabolism	1	7	0.14	1.02
Alanine and Aspartate Metabolism	1	8	0.12	0.89
Lysophospholipid	3	24	0.12	0.89
Methionine, Cysteine, SAM and Taurine Metabolism	2	17	0.12	0.84
Fatty Acid, Dicarboxylate	1	9	0.11	0.79
Polyamine Metabolism	1	9	0.11	0.79
Glycine, Serine and Threonine Metabolism	1	11	0.09	0.65

Tryptophan Metabolism	1	11	0.09	0.65
Fatty Acid Metabolism (Acyl Carnitine)	3	34	0.09	0.63
Leucine, Isoleucine and Valine Metabolism	2	24	0.08	0.59
Lysine Metabolism	1	12	0.08	0.59
Polyunsaturated Fatty Acid (n3 and n6)	1	12	0.08	0.59
Urea cycle; Arginine and Proline Metabolism	1	16	0.06	0.44

[1] All compounds; Significant (n) = 86, Detected (N) = 612, Significant/ detected (n/N) = 0.14

[2] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

Table 8: Pathways altered at d 135 in RES compared with CON.

Pathway Name	Pathway Metabolites			
	Significant (k)	Detected (m)	Significant/ Detected (k/m)	Enrichment [1] (k/m)/(n/N)
Bacterial/Fungal	1	1	1	8.5
Ketone Bodies	1	1	1	8.5
Phosphatidylglycerol	1	1	1	8.5
Riboflavin Metabolism	2	2	1	8.5
Histidine Metabolism	7	14	0.5	4.25
Pyrimidine Metabolism, Thymine containing	1	2	0.5	4.25
Secondary Bile Acid Metabolism	2	4	0.5	4.25
Hemoglobin and Porphyrin Metabolism	2	5	0.4	3.4
Glycerolipid Metabolism	1	3	0.33	2.83
Gamma-glutamyl Amino Acid	4	13	0.31	2.62
Tyrosine Metabolism	2	7	0.29	2.43
Glycine, Serine and Threonine Metabolism	3	11	0.27	2.32
Alanine and Aspartate Metabolism	2	8	0.25	2.13
Lysophospholipid	6	24	0.25	2.13
Methionine, Cysteine, SAM and Taurine Metabolism	4	17	0.24	2
Fatty Acid, Dicarboxylate	2	9	0.22	1.89
Polyamine Metabolism	2	9	0.22	1.89
Chemical	1	5	0.2	1.7
Glutathione Metabolism	2	10	0.2	1.7
Lysoplasmalogen	1	5	0.2	1.7
Phospholipid Metabolism	2	10	0.2	1.7
Urea cycle; Arginine and Proline Metabolism	3	16	0.19	1.59
Primary Bile Acid Metabolism	1	6	0.17	1.42
Food Component/Plant	2	13	0.15	1.31
Aminosugar Metabolism	1	7	0.14	1.21
Progestin Steroids	1	7	0.14	1.21
Phosphatidylcholine	2	17	0.12	1

TCA Cycle	1	9	0.11	0.94
Benzoate Metabolism	1	10	0.1	0.85
Plasmalogen	1	10	0.1	0.85
Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1	11	0.09	0.77
Purine Metabolism, (Hypo) Xanthine/Inosine containing	1	11	0.09	0.77
Tryptophan Metabolism	1	11	0.09	0.77
Lysine Metabolism	1	12	0.08	0.71
Polyunsaturated Fatty Acid (n3 and n6)	1	12	0.08	0.71
Pyrimidine Metabolism, Uracil containing	1	12	0.08	0.71
Glutamate Metabolism	1	13	0.08	0.65
Sphingolipid Metabolism	2	31	0.06	0.55
Leucine, Isoleucine and Valine Metabolism	1	24	0.04	0.35

[1] All compounds; Significant (n) = 72, Detected (N) = 612, Significant/ detected (n/N) = 0.12

[2] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

Table 9: Pathways altered at birth in RES compared with CON.

Pathway Name	Pathway Metabolites			Enrichment [1] (k/m)/(n/N)
	Significant (k)	Detected (m)	Significant/ Detected (k/m)	
Phosphatidylglycerol	1	1	1	9.56
Purine and Pyrimidine Metabolism	1	1	1	9.56
Tocopherol Metabolism	1	1	1	9.56
Phenylalanine Metabolism	2	3	0.67	6.37
Nucleotide Sugar	3	5	0.6	5.74
Guanidino and Acetamido Metabolism	1	2	0.5	4.78
Pyrimidine Metabolism, Orotate containing	1	2	0.5	4.78
Thiamine Metabolism	1	2	0.5	4.78
Lysophospholipid	10	24	0.42	3.98
Alanine and Aspartate Metabolism	3	8	0.38	3.59
Histidine Metabolism	4	14	0.29	2.73
Progestin Steroids	2	7	0.29	2.73
Secondary Bile Acid Metabolism	1	4	0.25	2.39
Phosphatidylcholine	4	17	0.24	2.25
Food Component/Plant	3	13	0.23	2.21
Lysoplasmalogen	1	5	0.2	1.91
Nicotinate and Nicotinamide Metabolism	2	10	0.2	1.91
Pyrimidine Metabolism, Cytidine containing	2	10	0.2	1.91
Primary Bile Acid Metabolism	1	6	0.17	1.59
Pyrimidine Metabolism, Uracil containing	2	12	0.17	1.59
Glutamate Metabolism	2	13	0.15	1.47
Fructose, Mannose and Galactose Metabolism	1	7	0.14	1.37
Urea cycle; Arginine and Proline Metabolism	2	16	0.12	1.2
Methionine, Cysteine, SAM and Taurine Metabolism	2	17	0.12	1.12
Fatty Acid, Dicarboxylate	1	9	0.11	1.06
Monoacylglycerol	1	9	0.11	1.06
Phosphatidylethanolamine	1	11	0.09	0.87

Purine Metabolism, (Hypo) Xanthine/Inosine containing	1	11	0.09	0.87
Purine Metabolism, Adenine containing	1	11	0.09	0.87
Tryptophan Metabolism	1	11	0.09	0.87
Fatty Acid Metabolism (Acyl Carnitine)	3	34	0.09	0.84
Sphingolipid Metabolism	2	31	0.06	0.62

[1] All compounds; Significant (n) = 64, Detected (N) = 612, Significant/ detected (n/N) = 0.10

[2] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

Table 10: Pathways altered at d 90 in OVER compared with CON.

Pathway Name	Pathway Metabolites			Enrichment [1] (k/m)/(n/N)
	Significant (k)	Detected (m)	Significant/ Detected (k/m)	
Acetylated Peptides	1	1	1	7.85
Fatty Acid, Amino	1	1	1	7.85
Pyrimidine Metabolism, Thymine containing	2	2	1	7.85
Pantothenate and CoA Metabolism	2	3	0.67	5.23
Nucleotide Sugar	3	5	0.6	4.71
Tyrosine Metabolism	4	7	0.57	4.48
Pentose Phosphate Pathway	1	2	0.5	3.92
Riboflavin Metabolism	1	2	0.5	3.92
Thiamine Metabolism	1	2	0.5	3.92
Vitamin B6 Metabolism	1	2	0.5	3.92
Alanine and Aspartate Metabolism	3	8	0.38	2.94
Dipeptide	1	3	0.33	2.62
Primary Bile Acid Metabolism	2	6	0.33	2.62
Glutathione Metabolism	3	10	0.3	2.35
Histidine Metabolism	4	14	0.29	2.24
Glycine, Serine and Threonine Metabolism	3	11	0.27	2.14
Purine Metabolism, Adenine containing	3	11	0.27	2.14
Tryptophan Metabolism	3	11	0.27	2.14
Secondary Bile Acid Metabolism	1	4	0.25	1.96
Food Component/Plant	3	13	0.23	1.81
Gamma-glutamyl Amino Acid	3	13	0.23	1.81
Glutamate Metabolism	3	13	0.23	1.81
Leucine, Isoleucine and Valine Metabolism	5	24	0.21	1.63
Ascorbate and Aldarate Metabolism	1	5	0.2	1.57
Chemical	1	5	0.2	1.57
Hemoglobin and Porphyrin Metabolism	1	5	0.2	1.57
Urea cycle; Arginine and Proline Metabolism	3	16	0.19	1.47

Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	2	11	0.18	1.43
Long Chain Fatty Acid	2	12	0.17	1.31
Fructose, Mannose and Galactose Metabolism	1	7	0.14	1.12
Methionine, Cysteine, SAM and Taurine Metabolism	2	17	0.12	0.92
TCA Cycle	1	9	0.11	0.87
Benzoate Metabolism	1	10	0.1	0.78
Nicotinate and Nicotinamide Metabolism	1	10	0.1	0.78
Purine Metabolism, (Hypo) Xanthine/Inosine containing	1	11	0.09	0.71
Lysine Metabolism	1	12	0.08	0.65
Lysophospholipid	2	24	0.08	0.65
Pyrimidine Metabolism, Uracil containing	1	12	0.08	0.65
Sphingolipid Metabolism	2	31	0.06	0.51
Phosphatidylcholine	1	17	0.06	0.46

[1] All compounds; Significant (n) = 78, Detected (N) = 612, Significant/ detected (n/N) = 0.13

[2] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

Table 11: Pathways altered at d 135 in OVER compared with CON.

Pathway Name	Pathway Metabolites			Enrichment [1] (k/m)/(n/N)
	Significant (k)	Detected (m)	Significant/ Detected (k/m)	
Bacterial/Fungal	1	1	1	22.67
Pyrimidine Metabolism, Orotate containing	1	2	0.5	11.33
Fatty Acid, Dicarboxylate	2	9	0.22	5.04
Endocannabinoid	1	5	0.2	4.53
Hemoglobin and Porphyrin Metabolism	1	5	0.2	4.53
Lysoplasmalogen	1	5	0.2	4.53
Nicotinate and Nicotinamide Metabolism	2	10	0.2	4.53
Methionine, Cysteine, SAM and Taurine Metabolism	3	17	0.18	4
Histidine Metabolism	2	14	0.14	3.24
Tyrosine Metabolism	1	7	0.14	3.24
Alanine and Aspartate Metabolism	1	8	0.12	2.83
Leucine, Isoleucine and Valine Metabolism	3	24	0.12	2.83
Phosphatidylcholine	2	17	0.12	2.67
Benzoate Metabolism	1	10	0.1	2.27
Glutathione Metabolism	1	10	0.1	2.27
Phosphatidylethanolamine	1	11	0.09	2.06
Tryptophan Metabolism	1	11	0.09	2.06
Food Component/Plant	1	13	0.08	1.74
Urea cycle; Arginine and Proline Metabolism	1	16	0.06	1.42

[1] All compounds; Significant (n) = 27, Detected (N) = 612, Significant/ detected (n/N) = 0.04

[2] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

Table 12: Pathways altered at birth in OVER compared with CON.

Pathway Name	Pathway Compounds			Enrichment [1] (k/m)/(n/N)
	Significant (k)	Detected (m)	Significant/ Detected (k/m)	
Acetylated Peptides	1	1	1	6
Advanced Glycation End-product	1	1	1	6
Fatty Acid Metabolism (Acyl Choline)	1	1	1	6
Phosphatidylglycerol	1	1	1	6
Riboflavin Metabolism	2	2	1	6
Tocopherol Metabolism	1	1	1	6
Vitamin A Metabolism	1	1	1	6
Phosphatidylethanolamine	10	11	0.91	5.45
Alanine and Aspartate Metabolism	6	8	0.75	4.5
Inositol Metabolism	2	3	0.67	4
Lysophospholipid	13	24	0.54	3.25
Fatty Acid Metabolism (also BCAA Metabolism)	1	2	0.5	3
Phosphatidylinositol	1	2	0.5	3
Polyamine Metabolism	4	9	0.44	2.67
Lysoplasmalogen	2	5	0.4	2.4
Phenylalanine Metabolism	1	3	0.33	2
Fatty Acid Metabolism (Acyl Carnitine)	11	34	0.32	1.94
Aminosugar Metabolism	2	7	0.29	1.71
Glycine, Serine and Threonine Metabolism	3	11	0.27	1.64
Methionine, Cysteine, SAM and Taurine Metabolism	4	17	0.24	1.41
Phosphatidylcholine	4	17	0.24	1.41
Chemical	1	5	0.2	1.2
Hemoglobin and Porphyrin Metabolism	1	5	0.2	1.2
Medium Chain Fatty Acid	1	5	0.2	1.2
Nucleotide Sugar	1	5	0.2	1.2
Plasmalogen	2	10	0.2	1.2
Urea cycle; Arginine and Proline Metabolism	3	16	0.19	1.12

Tryptophan Metabolism	2	11	0.18	1.09
Lysine Metabolism	2	12	0.17	1
Gamma-glutamyl Amino Acid	2	13	0.15	0.92
Fatty Acid, Dicarboxylate	1	9	0.11	0.67
Benzoate Metabolism	1	10	0.1	0.6
Ceramides	1	10	0.1	0.6
Glutathione Metabolism	1	10	0.1	0.6
Phospholipid Metabolism	1	10	0.1	0.6
Sphingolipid Metabolism	3	31	0.1	0.58
Glycolysis, Gluconeogenesis, and Pyruvate Metabolism	1	11	0.09	0.55
Purine Metabolism, (Hypo)Xanthine/Inosine containing	1	11	0.09	0.55
Polyunsaturated Fatty Acid (n3 and n6)	1	12	0.08	0.5
Pyrimidine Metabolism, Uracil containing	1	12	0.08	0.5
Food Component/Plant	1	13	0.08	0.46
Glutamate Metabolism	1	13	0.08	0.46
Histidine Metabolism	1	14	0.07	0.43

[1] All compounds; Significant (n) = 102, Detected (N) = 612, Significant/ detected (n/N) = 0.17

[2] Offspring from ewes fed a CON (100% NRC), RES (60% NRC), or OVER (140% NRC) diet. Ewe diet was based on the NRC requirement for pregnant ewes bearing twins and began at d 30 of gestation. At d 90 or 135 of gestation, ewes were euthanized and a hysterectomy was performed to acquire LD samples. Lamb LD was collected within 24 h of birth from ewes allowed to undergo parturition.

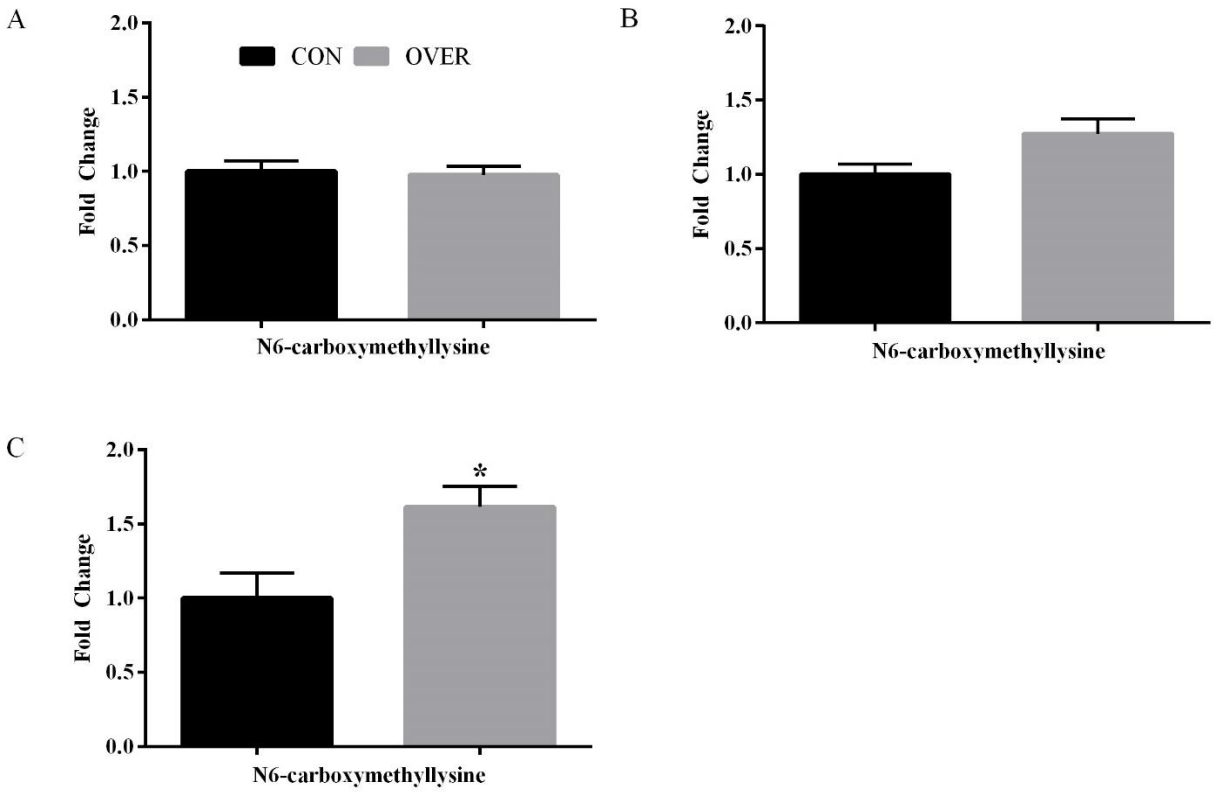

Figure 6: Over-feeding during gestation increased offspring advanced glycation end products at birth. Offspring metabolites from advanced glycation end products were altered in OVER compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

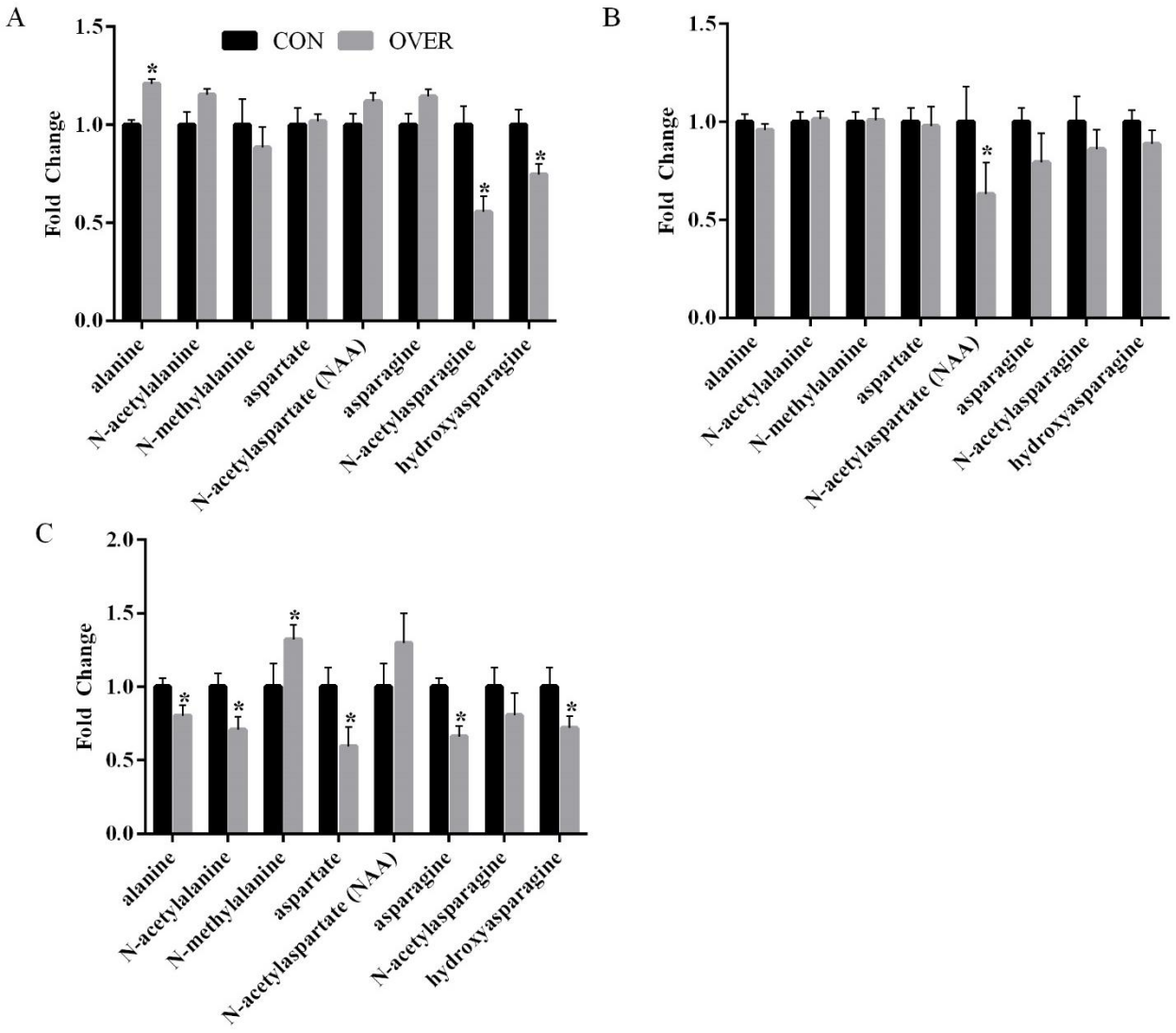

Figure 7: Over-feeding during gestation alters offspring alanine and aspartate metabolism at multiple time points of gestation. Offspring metabolites involved in alanine and aspartate metabolism were altered in OVER compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

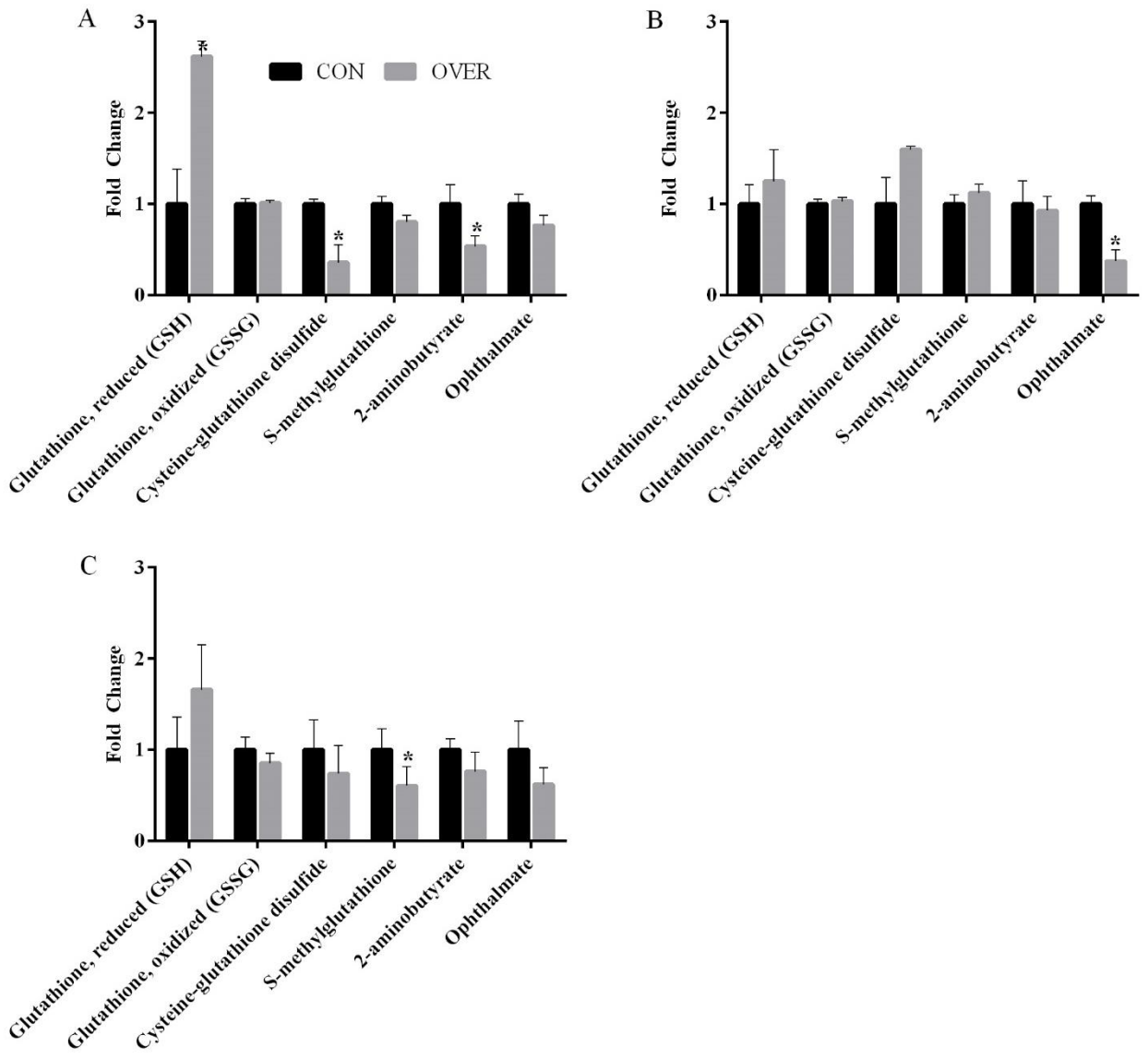

Figure 8: Over-feeding during gestation alters offspring glutathione metabolism at multiple time points of gestation. Offspring metabolites involved in glutathione metabolism were altered in OVER compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

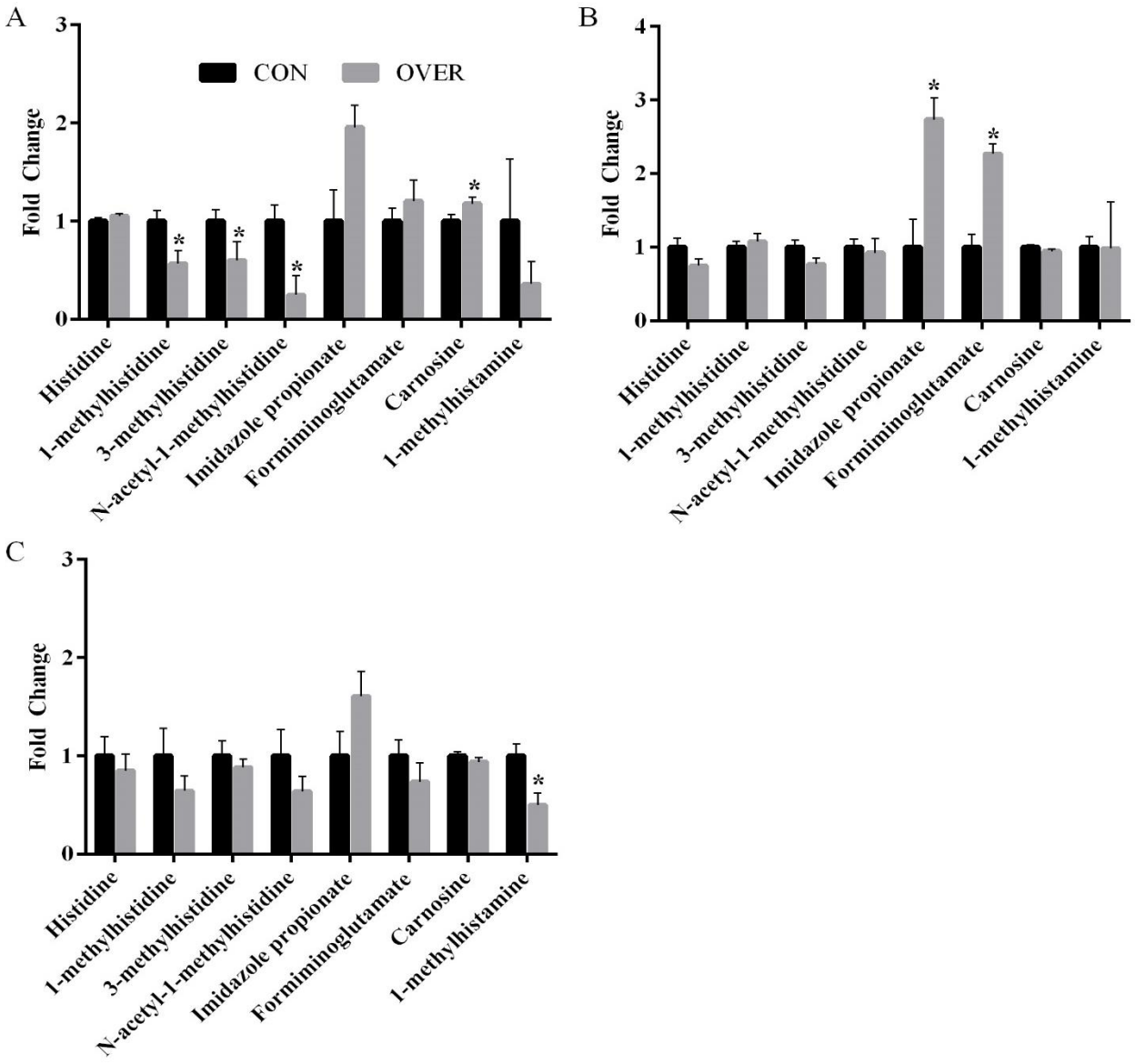

Figure 9: Over-feeding during gestation alters offspring histidine metabolism at multiple time points of gestation. Offspring metabolites involved histidine metabolism were altered in OVER compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

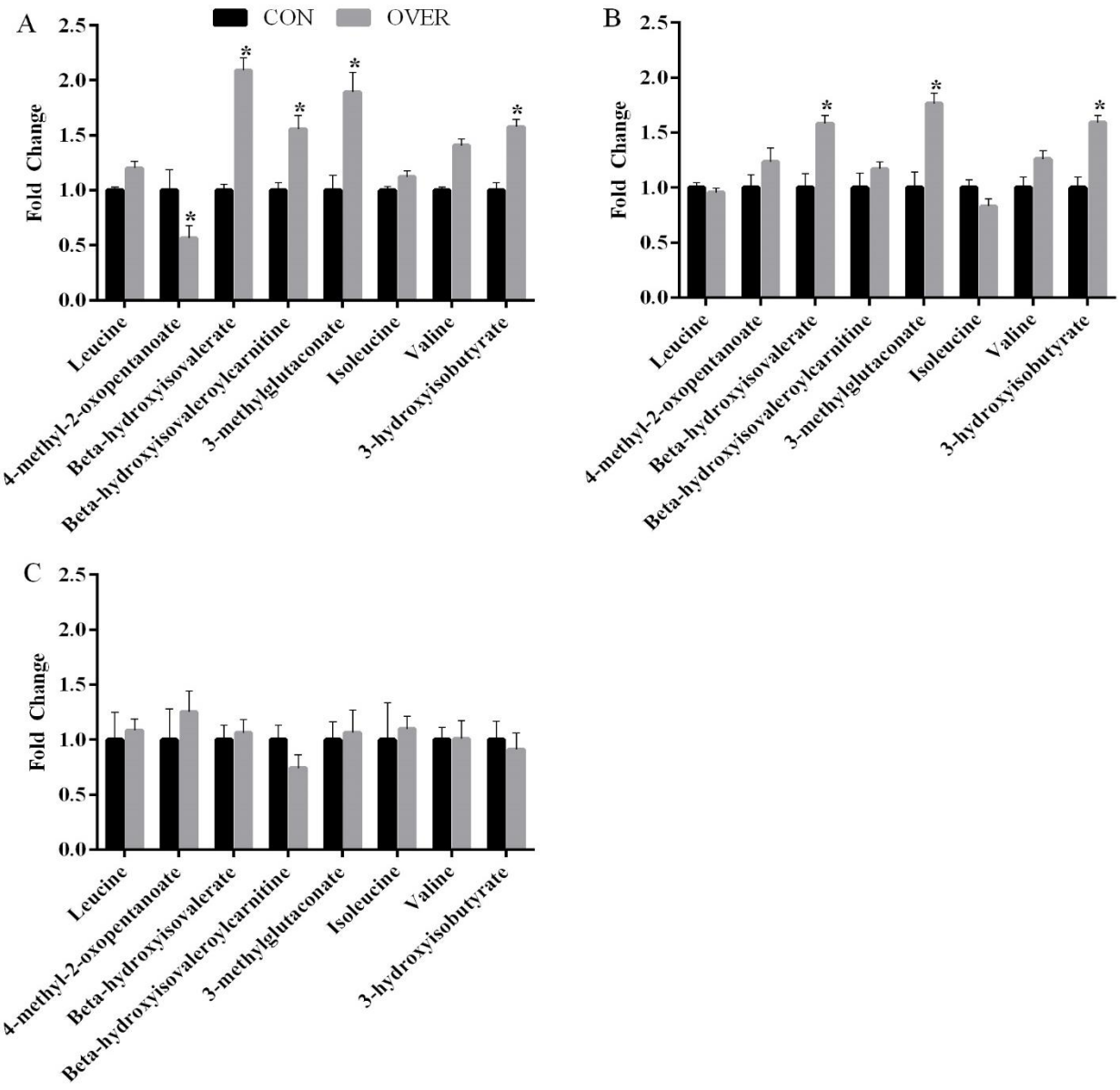

Figure 10: Over-feeding during gestation alters offspring leucine, isoleucine, and valine metabolism at multiple time points of gestation. Offspring metabolites involved in leucine, isoleucine, and valine metabolism were altered in OVER compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

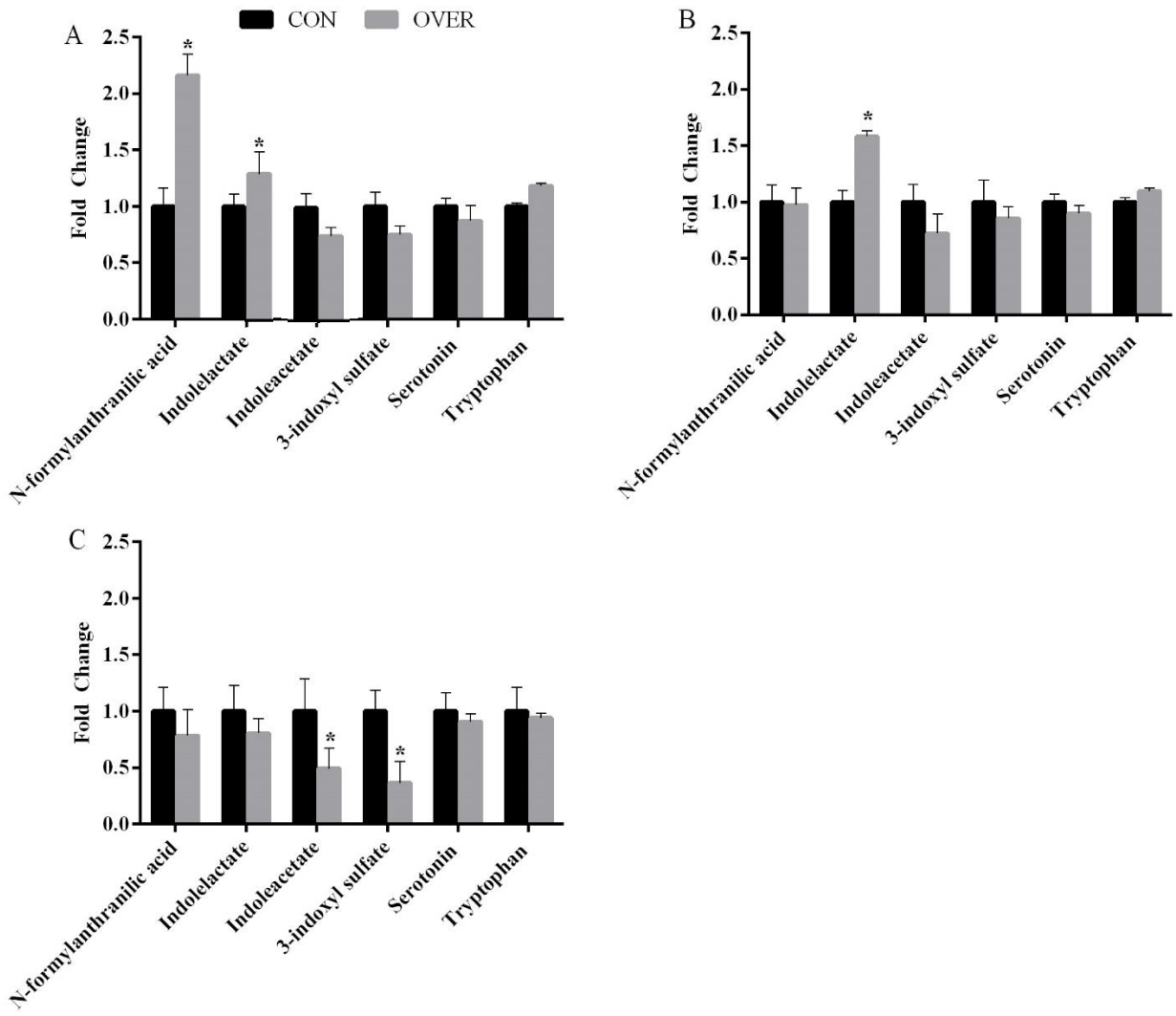

Figure 11: Over-feeding during gestation alters offspring tryptophan metabolism at multiple time points of gestation. Offspring metabolites involved in tryptophan metabolism were altered in OVER compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

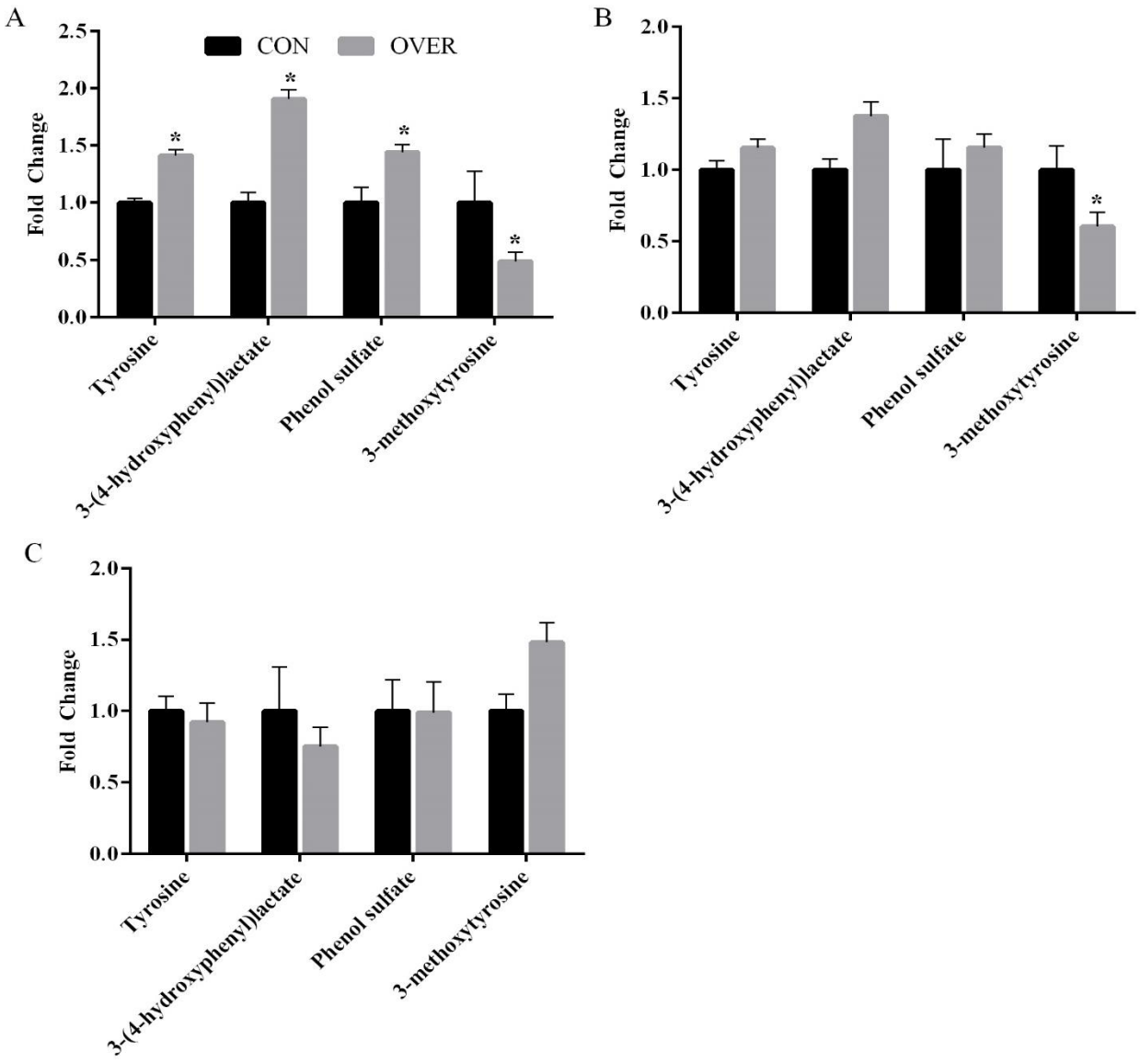

Figure 12: Over-feeding during gestation alters offspring tyrosine metabolism at multiple time points of gestation. Offspring metabolites involved in tyrosine metabolism were altered in OVER compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

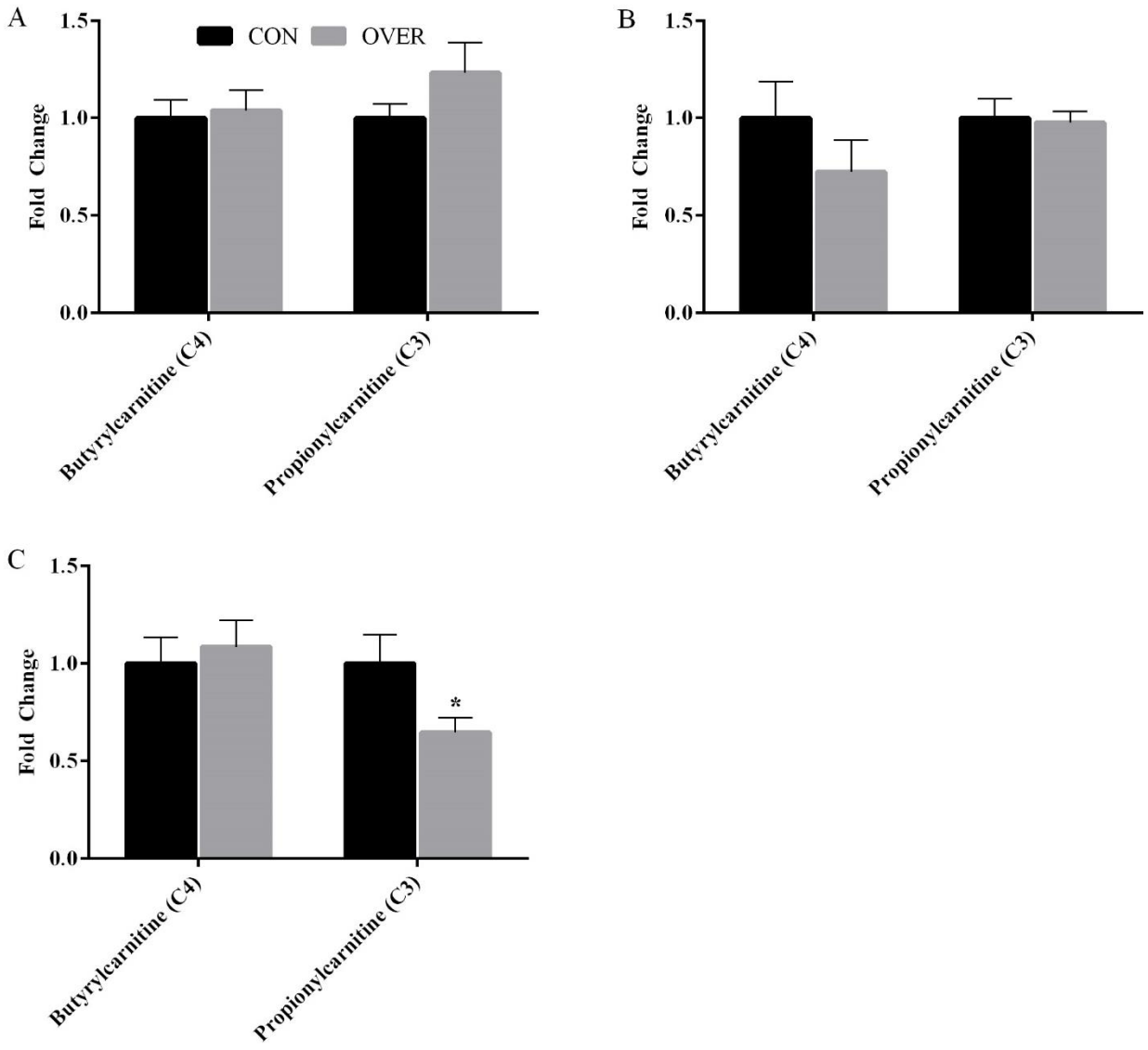

Figure 13: Over-feeding during gestation alters offspring fatty acid and branched chain amino acid metabolism at birth. Offspring metabolites involved in fatty acid and branched chain amino acid metabolism were altered in OVER compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

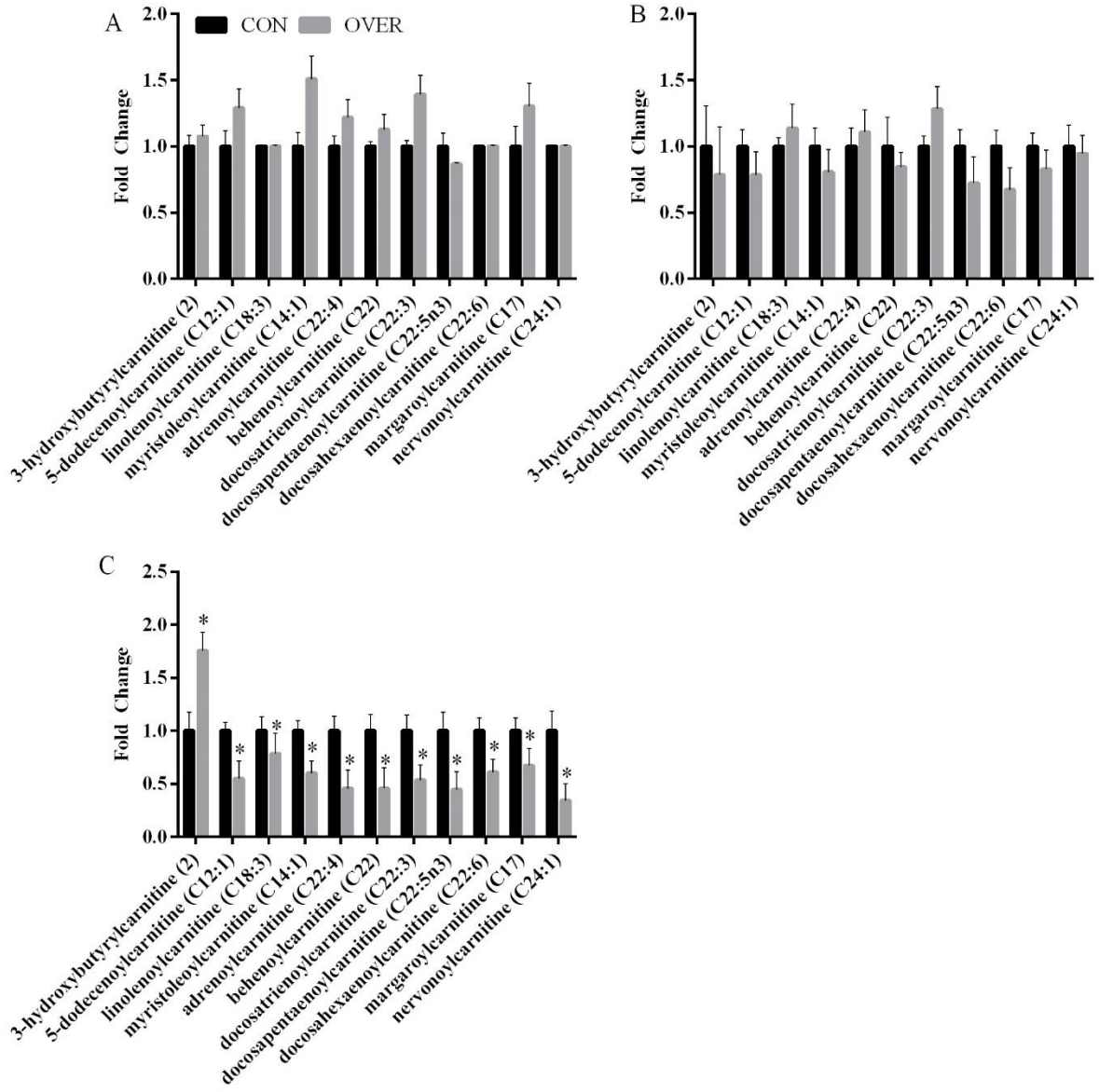

Figure 14: Over-feeding during gestation alters offspring fatty acid and acyl carnitine metabolism at birth. Offspring metabolites involved in fatty acid and acyl carnitine metabolism were altered in OVER compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

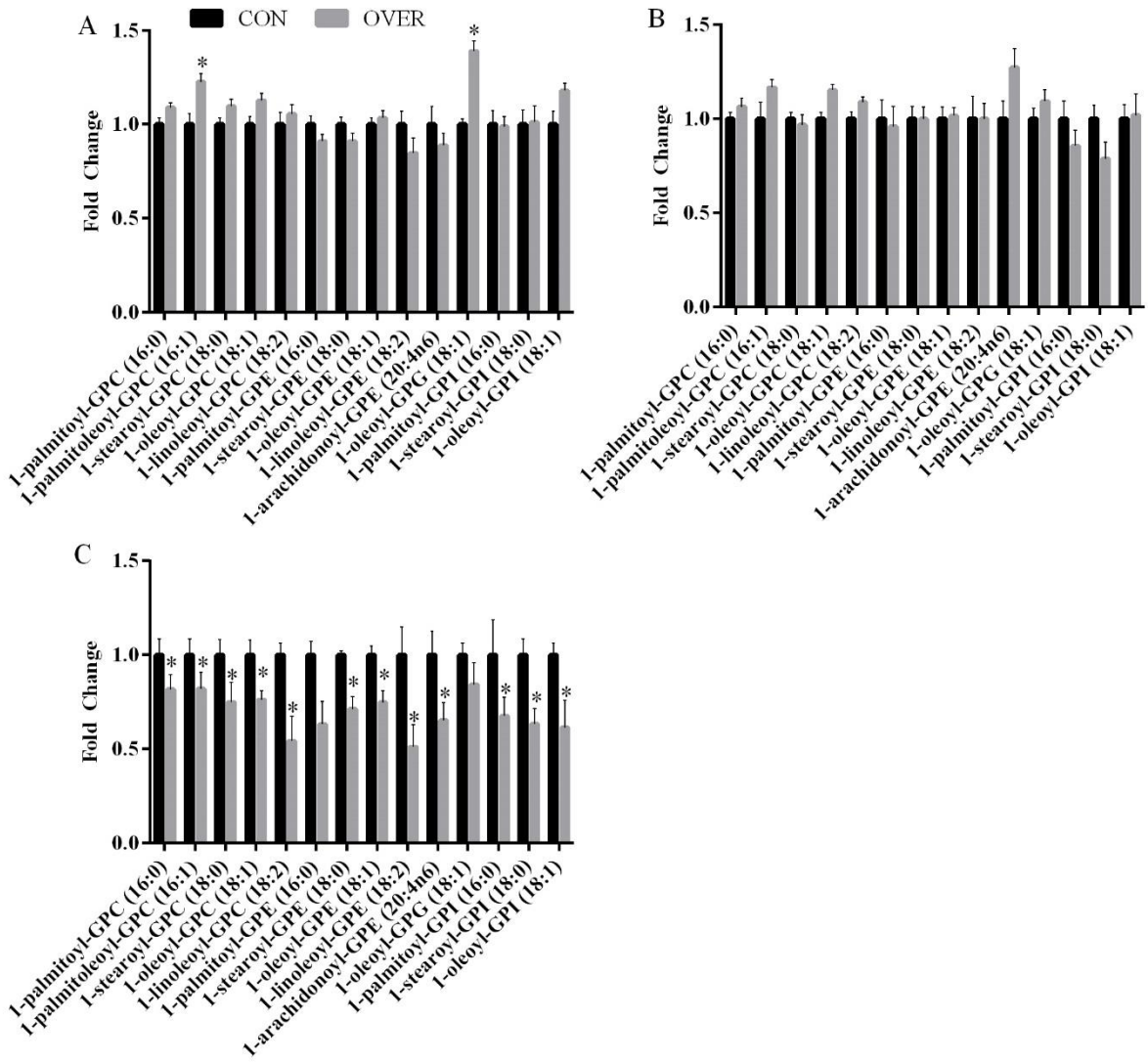

Figure 15: Over-feeding during gestation alters offspring lysophospholipid metabolism at multiple time points of gestation. Offspring metabolites involved in lysophospholipid metabolism were altered in OVER compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

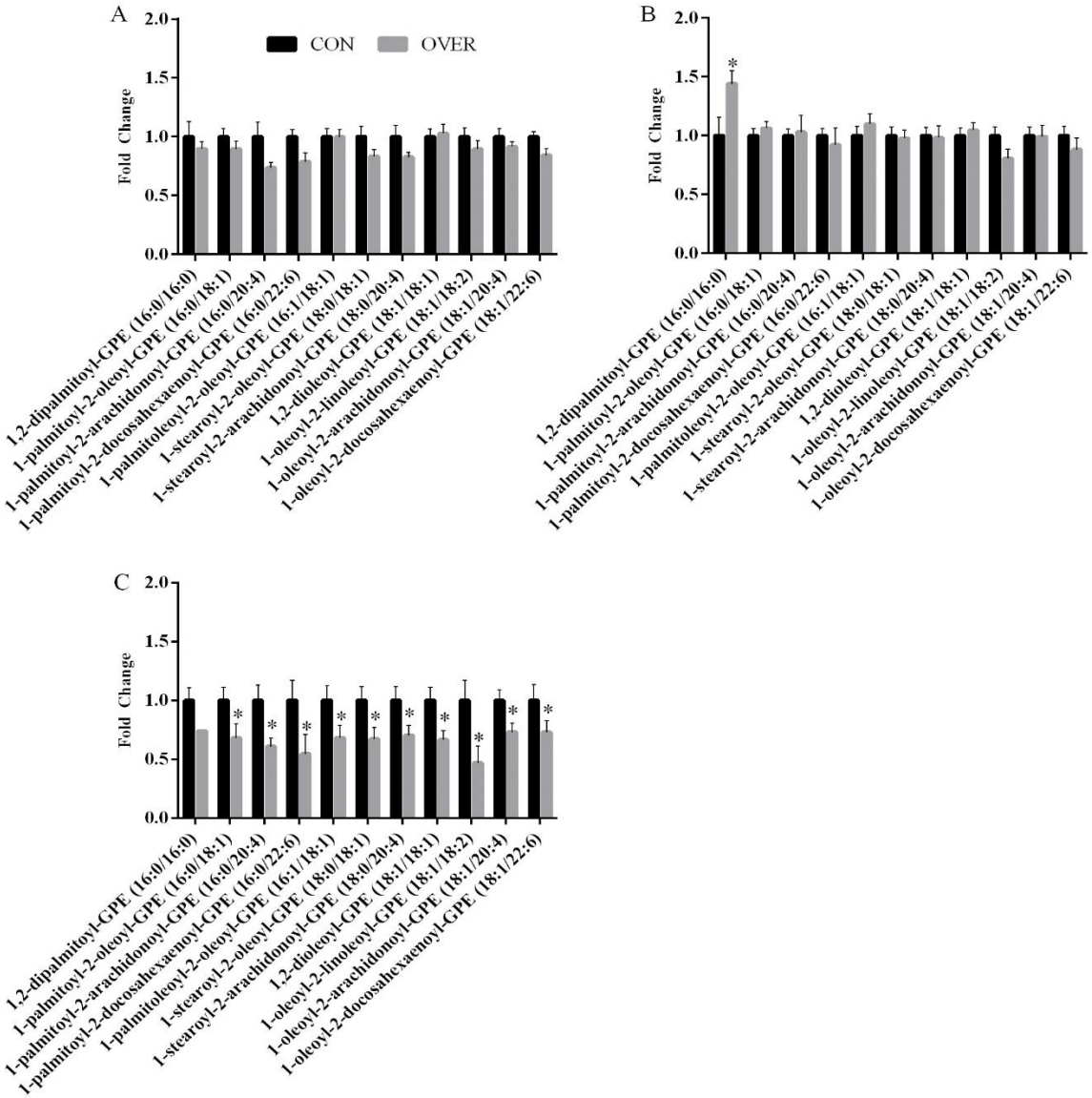

Figure 16: Over-feeding during gestation alters offspring phosphatidylethanolamine metabolism at multiple time points of gestation. Offspring metabolites involved in phosphatidylethanolamine metabolism were altered in OVER compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

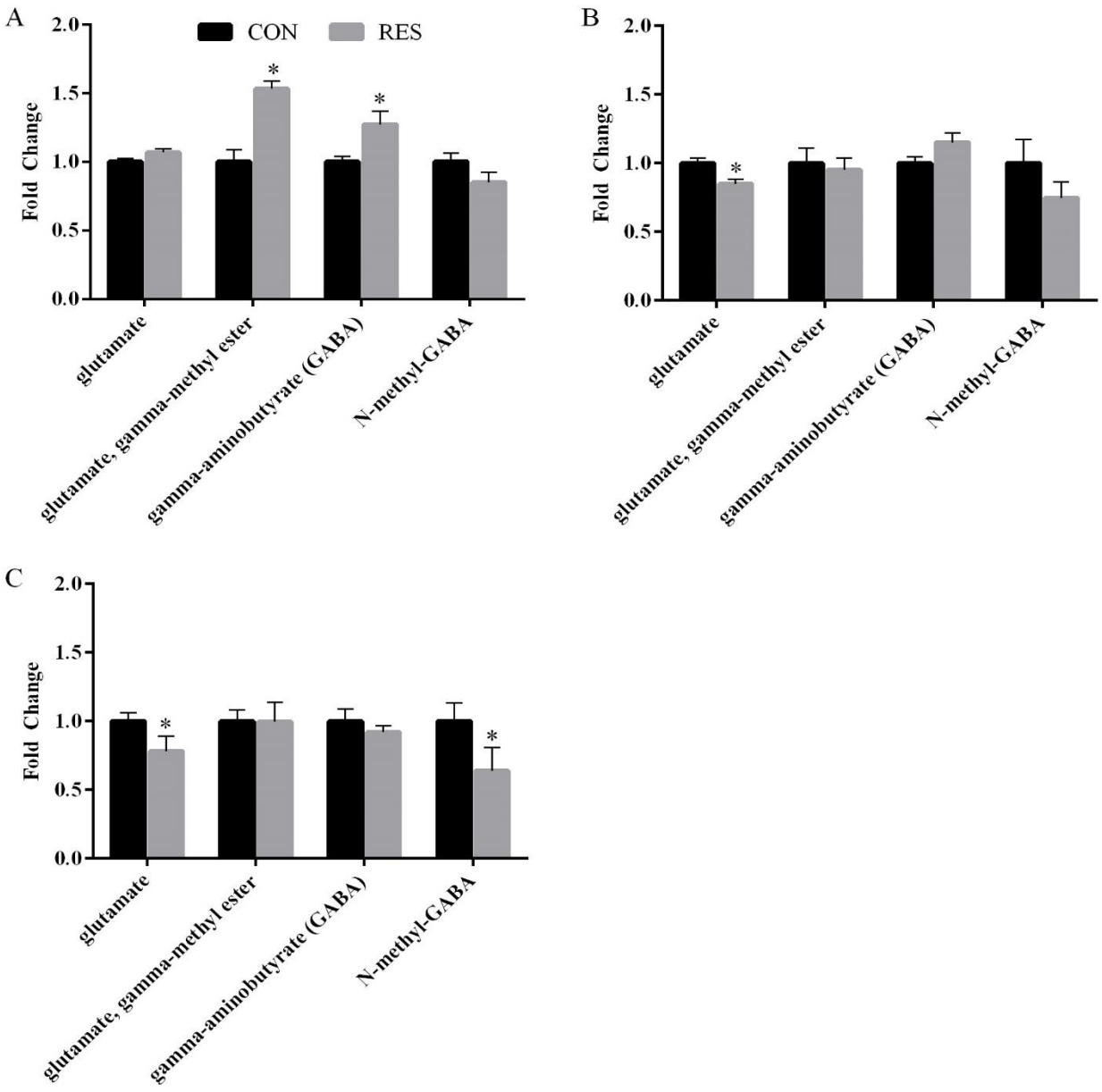

Figure 17: Restricted-feeding during gestation alters offspring glutamate metabolism at multiple time points of gestation. Offspring metabolites involved in glutamate metabolism were altered in RES compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

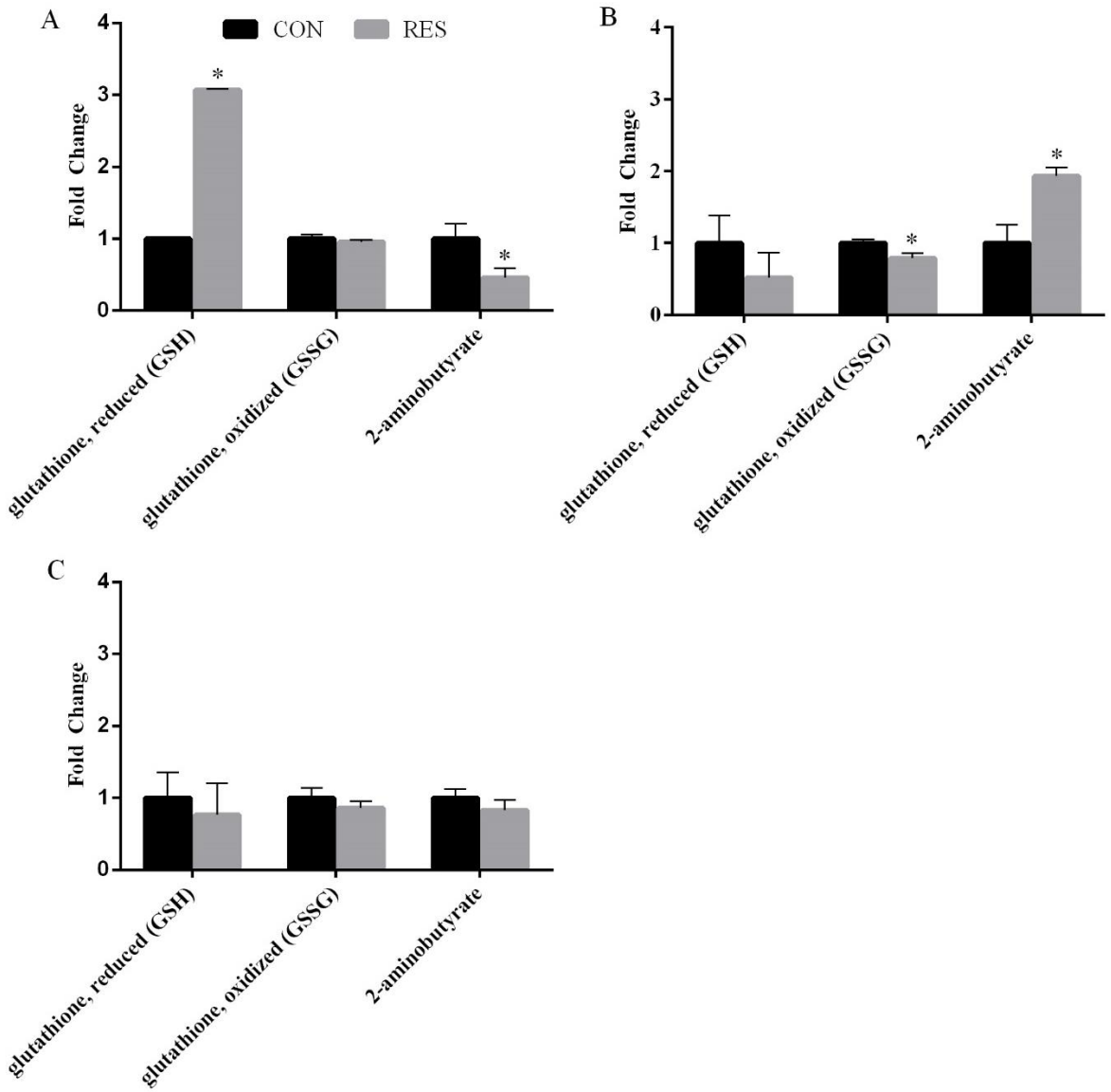

Figure 18: Restricted-feeding during gestation alters offspring glutathione metabolism at multiple time points of gestation. Offspring metabolites involved in glutathione metabolism were altered in RES compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

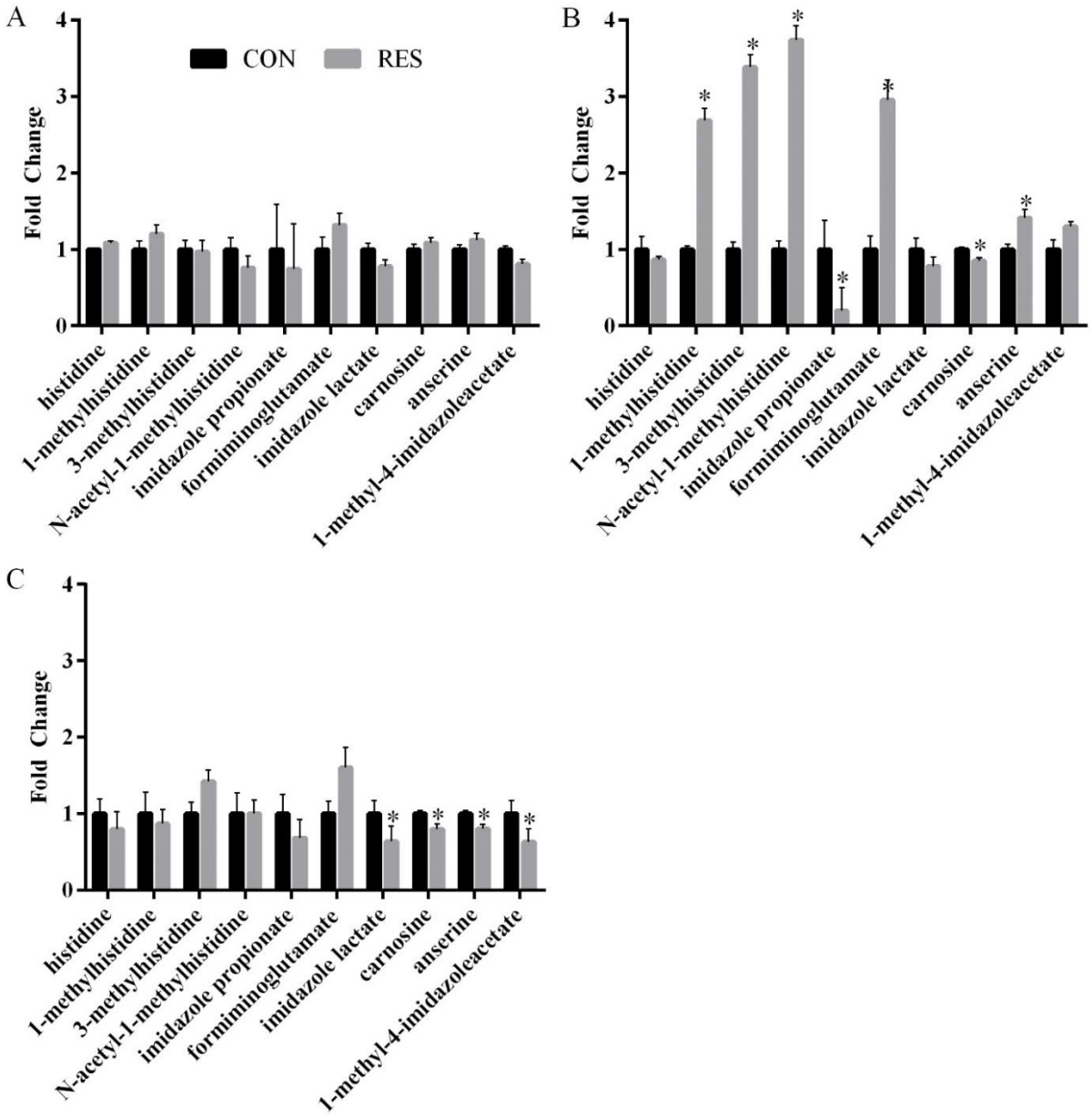

Figure 19: Restricted-feeding during gestation alters offspring histidine metabolism at multiple time points of gestation. Offspring metabolites involved in histidine metabolism were altered in RES compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

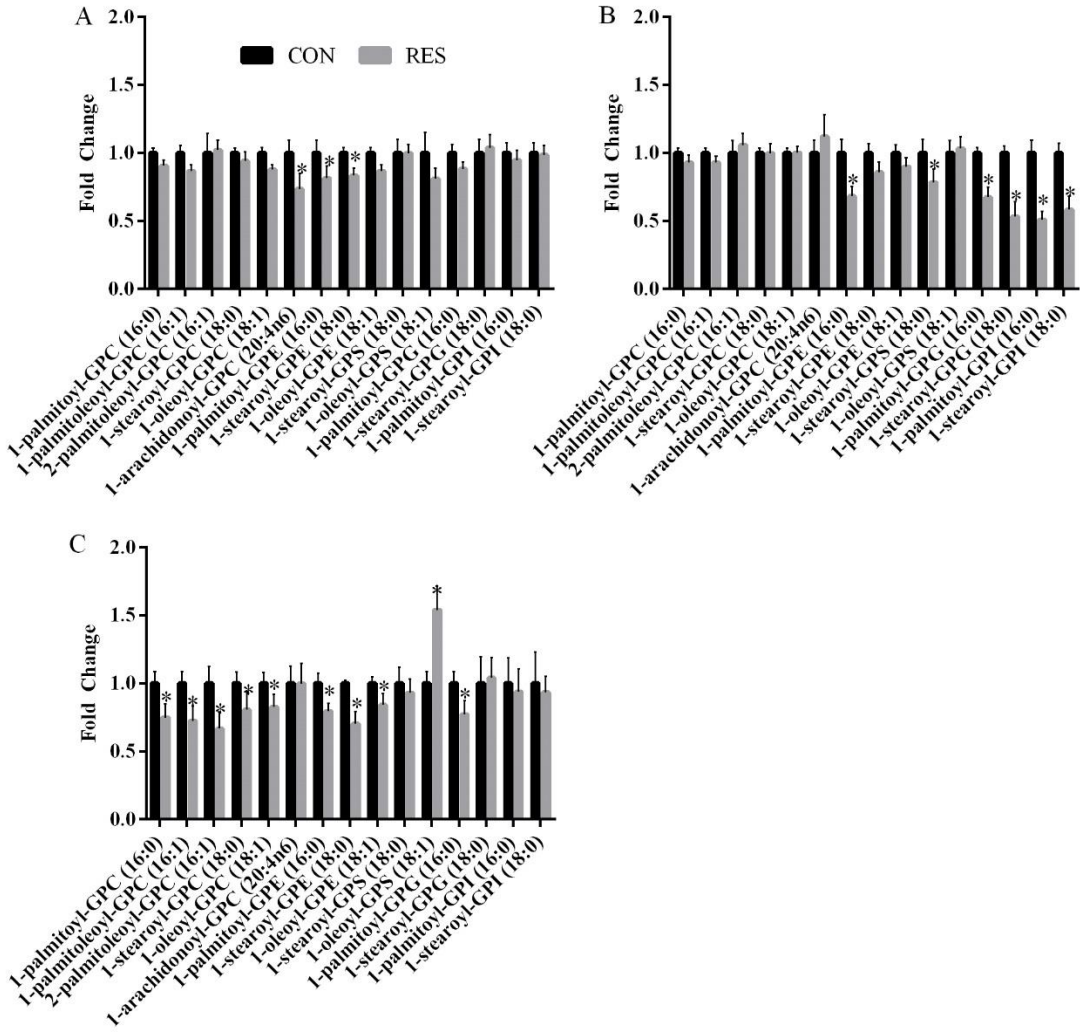

Figure 20: Restricted-feeding during gestation alters offspring lysophospholipid metabolism at multiple time points of gestation. Offspring metabolites involved in lysophospholipid metabolism were altered in RES compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

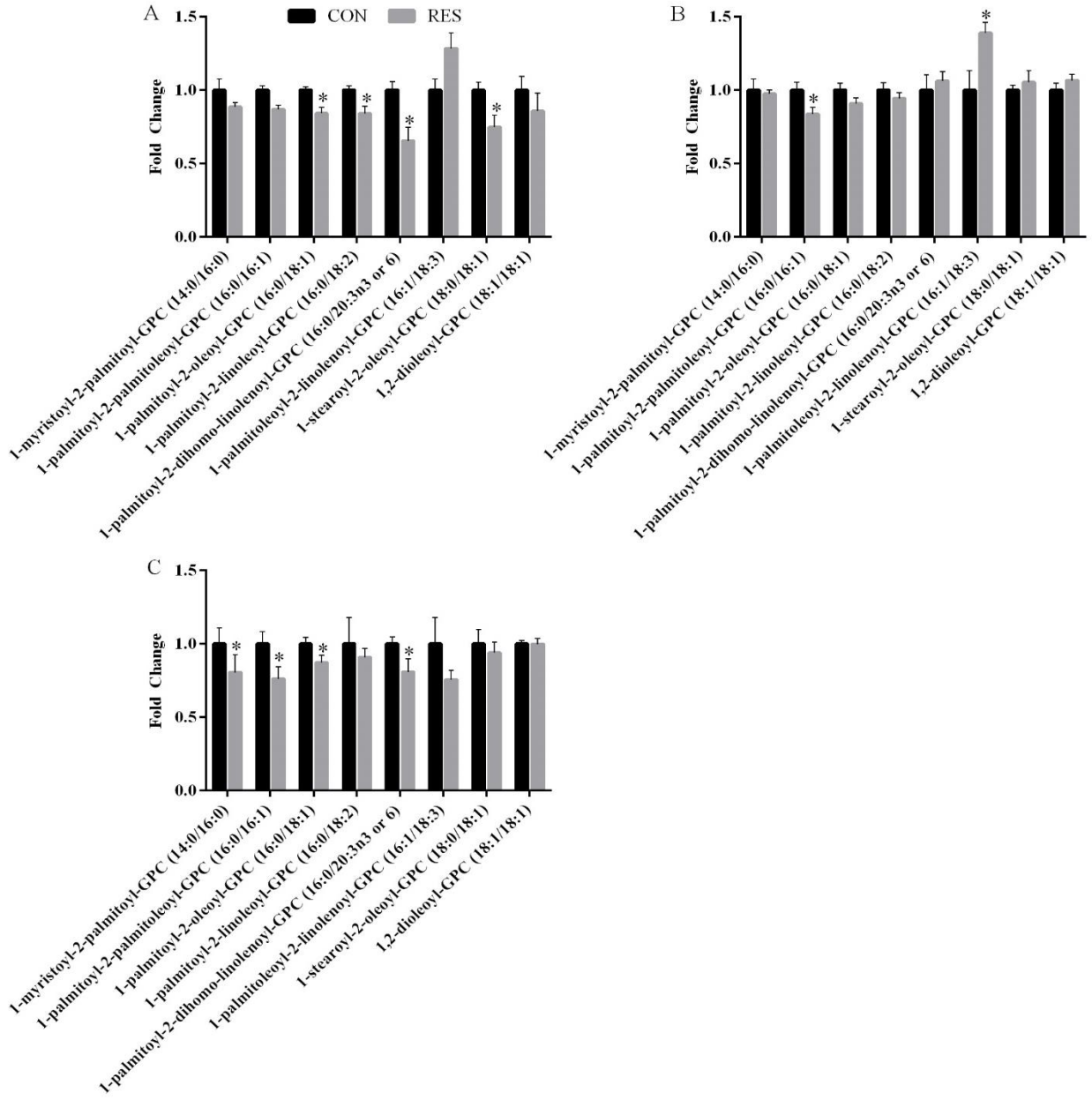

Figure 21: Restricted-feeding during gestation alters offspring phosphatidylcholine metabolism at multiple time points of gestation. Offspring metabolites involved in phosphatidylcholine metabolism were altered in RES compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

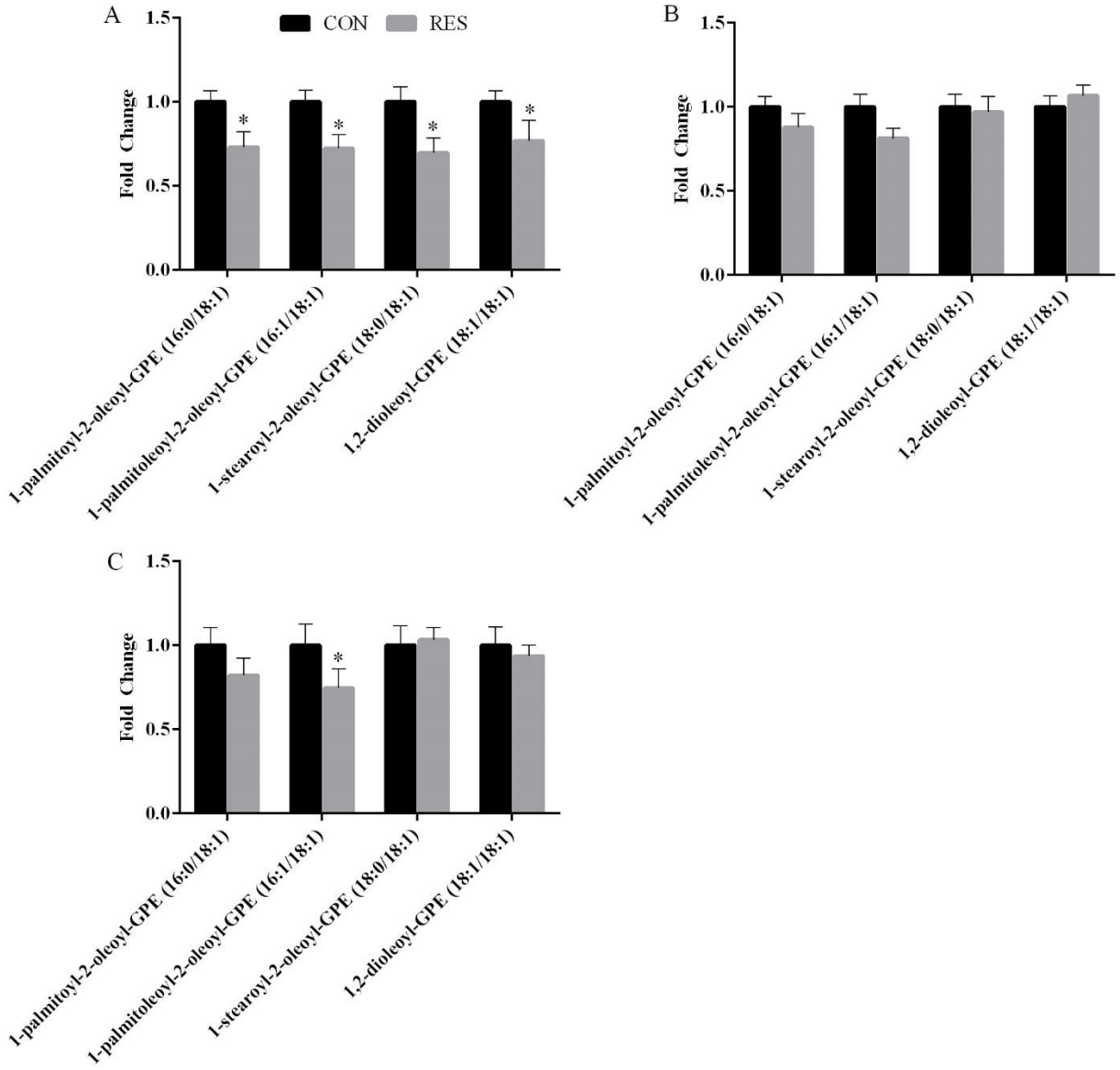

Figure 22: Restricted-feeding during gestation alters offspring phosphatidylethanolamine metabolism at multiple time points of gestation. Offspring metabolites involved in phosphatidylethanolamine metabolism were altered in RES compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change. * denotes $P \leq 0.05$ compared with CON.

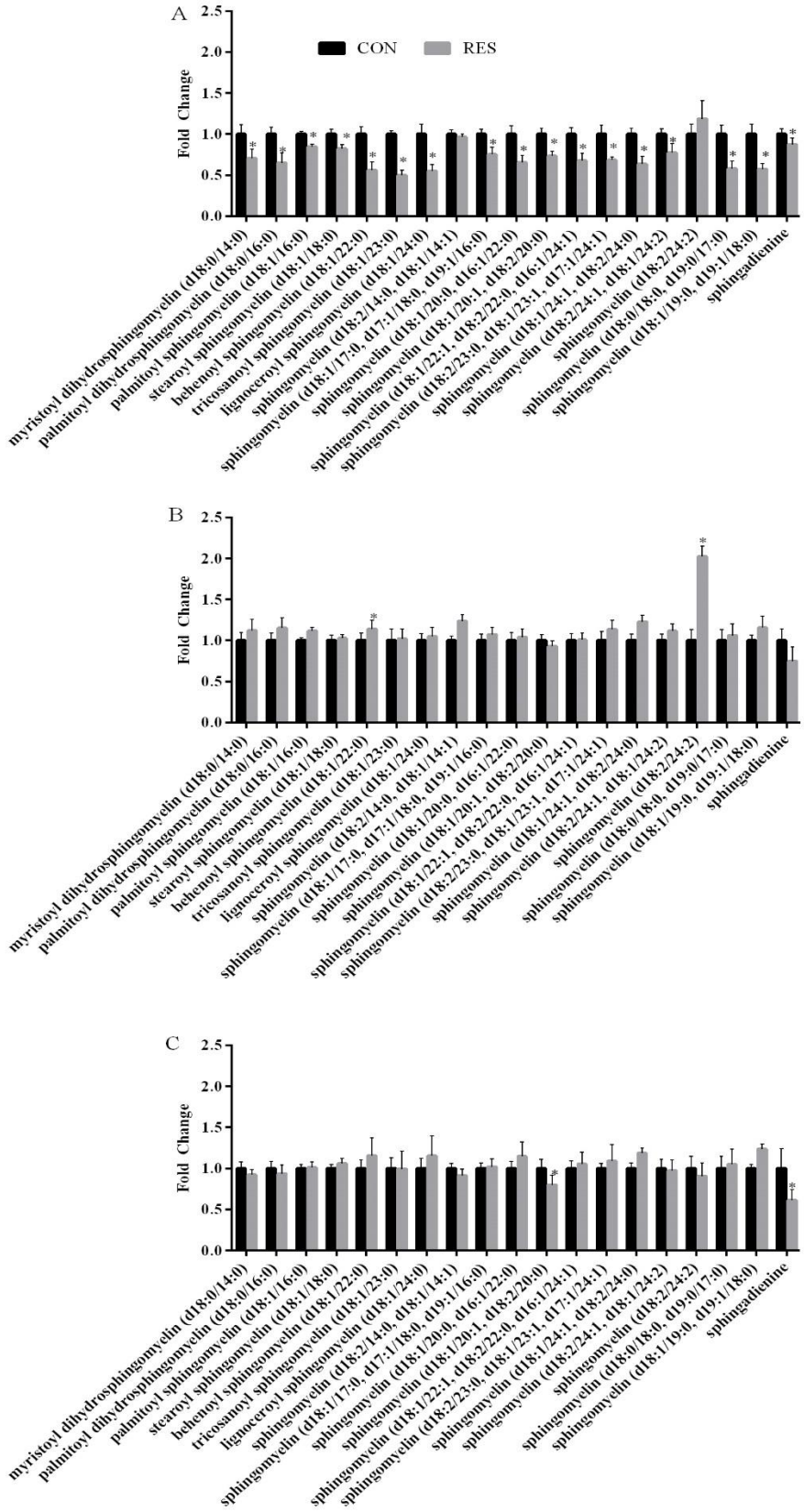
A
B
C
CON
RES
Fold Change
0.0
0.5
1.0
1.5
2.0
2.5
myristoyl dihydrosphingomyelin (d18:0/14:0)
palmitoyl dihydrosphingomyelin (d18:0/16:0)
palmitoyl sphingomyelin (d18:1/16:0)
stearoyl sphingomyelin (d18:1/18:0)
behenoyl sphingomyelin (d18:1/22:0)
tricosanoyl sphingomyelin (d18:1/23:0)
lignoceroyl sphingomyelin (d18:1/24:0)
sphingomyelin (d18:1/17:0, d17:1/18:0, d19:1/16:0)
sphingomyelin (d18:2/14:0, d18:1/14:1)
sphingomyelin (d18:1/20:0, d19:1/16:0)
sphingomyelin (d18:1/20:0, d16:1/22:0)
sphingomyelin (d18:1/20:1, d18:2/20:0)
sphingomyelin (d18:1/22:1, d18:2/22:0, d16:1/24:1)
sphingomyelin (d18:2/23:0, d18:1/23:1, d17:1/24:1)
sphingomyelin (d18:1/24:1, d18:2/24:0)
sphingomyelin (d18:2/24:1, d18:1/24:2)
sphingomyelin (d18:0/18:0, d19:0/17:0)
sphingomyelin (d18:1/19:0, d19:1/18:0)
sphingadienine

Figure 23: Restricted-feeding during gestation alters offspring sphingolipid metabolism at birth. Offspring metabolites involved in sphingolipid metabolism were altered in RES compared with CON. LD collected from offspring born to ewes fed CON, RES, or OVER diets at d 90 (A), 135 (B) of gestation, or within 24 hours of birth (C) was analyzed by UPLC-MS/MS. Data are presented as fold change ± standard error. * denotes $P \leq 0.05$ compared with CON.

DISCUSSION

When assessing the effects of poor maternal nutrition during gestation on offspring development, many research groups focus on either restricted- (Vonnahme et al., 2003; Daniel et al., 2007; Ford et al., 2007; Ge et al., 2013) or over-feeding (Zhu et al., 2008; Tong et al., 2009; Caton et al., 2009; Long et al., 2010; Yan et al., 2011). Our model is unique since it allows for comparison of both restricted- and over-nutrition at multiple time points during gestation (Pillai et al., 2017). Additionally, the multiple prenatal and birth time points allow us to assess changes to offspring development and metabolism over time. Restricted- and over-feeding can result in similar phenotypic changes to the offspring, such as changes in CSA of muscle fibers (Reed et al., 2014). However, the mechanisms behind these changes are often different, due to the influence of the maternal diet (Hoffman et al., 2016). One mechanism that can promote these changes is alterations to offspring metabolism (Hoffman et al., 2017). Previous studies have determined that metabolism is a key factor that determines if a cell will proliferate, differentiate, or remain quiescent (Ochocki and Simon, 2013; Shyh-Chang et al., 2013; Hu et al., 2016). Energy metabolism and nutrient sensing help regulate stem cell function and tissue development during gestation (Ochocki and Simon, 2013; Shyh-Chang et al., 2013; Hu et al., 2016). This is especially important for muscle, since gestation is a critical window of development for skeletal muscle formation and requires the coordinated progression of cell proliferation, cell cycle exit, and differentiation (Braun et al., 2011).

Changes to the intrauterine environment and offspring metabolism in response to poor maternal nutrition can alter the concentrations of hormones and growth factors, ultimately affecting metabolism. Specifically, lambs exposed to under- and over-nutrition during gestation demonstrated decreased serum concentrations of IGF1 and IGF binding protein 3 at birth

(Hoffman et al., 2014). Further, at 3 mo of age, leptin concentrations were increased in offspring from over-fed ewes compared with restricted-fed ewes (Hoffman et al., 2016). In the same study, average insulin concentrations between birth and 3 mo of age were increased in offspring from over-fed and restricted-fed ewes compared with controls (Hoffman et al., 2016). Exposure to maternal undernutrition also altered glucose tolerance in the offspring (Ford et al., 2007). Specifically, at d 63 of age, lambs from restricted-fed mothers had greater area under the curve for glucose and insulin compared with controls. These changes to hormone and growth factor concentrations have the potential to influence the formation and development of muscle fibers during gestation (Baum et al., 1996; Sotiropoulos et al., 2006; Velloso et al., 2008).

Circulating factors and hormone concentrations can impact muscle fiber formation and growth by increasing muscle mass and regulating fiber formation (Baum et al., 1996; Sotiropoulos et al., 2006; Velloso et al., 2008). These circulating factors may also be influencing the muscle phenotype by altering stem cell populations. Studies support that poor maternal nutrition can negatively impact offspring stem cells. In mice, exposure to both maternal under-nutrition and early postnatal over-nutrition decreased the number of skeletal muscle precursor cells (Woo et al., 2011). In the absence of cultured skeletal muscle precursor cells from lambs, C2C12 myoblasts can be used to assess how serum from offspring exposed to poor maternal nutrition influences myoblast proliferation, differentiation, and metabolism. Despite evidence which supports changes to hormones and growth factors in offspring serum in response to poor maternal nutrition, serum collected at birth from offspring exposed to under- or over-nutrition had no effect on myoblast proliferation, fiber fusion index, or fiber diameter. Other factors such as genetics and epigenetics can regulate muscle development (Peñagaricano et al., 2014; Kalbe et al., 2017). Many experiments demonstrate that poor maternal nutrition influences gene

expression in muscle. For example, Kalbe et al., (2017) found that exposure to maternal increased and restricted protein diets altered the mRNA expression of myogenic regulatory factors and IGFs. Another experiment demonstrated that maternal high-starch diet induces gene expression changes in the skeletal muscle and adipose of lambs (Peñagaricano et al., 2014). Specifically, genes involved in myogenic differentiation, glycolysis, gluconeogenesis, and the insulin signaling pathway were differentially expressed (Peñagaricano et al., 2014). These alterations to gene expression in response to poor maternal nutrition may be causing changes to muscle development along with changes to hormone and growth factor concentrations in serum. Additionally, the birth time point for serum collection may be a reason that there are no changes to proliferation or differentiation. Many studies examine changes to hormone concentrations at postnatal time points when the animal is more mature (Ford et al., 2007; Hoffman et al., 2014, 2016). Collecting serum from lambs exposed to poor maternal nutrition during gestation at later time points may result in different findings.

In addition to assessing whether serum from offspring from over- and restricted-fed ewes could influence C2C12 proliferation and differentiation, we analyzed whether the serum could influence myoblast metabolism. The serum collected from offspring exposed to restricted maternal diet altered the mitochondrial respiration of C2C12 myoblasts. Specifically, offspring from restricted-fed ewes exhibited increased proton leak compared with controls. Proton pumping across the mitochondrial membrane and proton leak contribute to a cycle that dissipates redox energy (Rolfe et al., 1999). Additionally, the mitochondrial proton cycle contributes to 15% of the standard metabolic rate in skeletal muscle (Rolfe et al., 1999). Increased proton leak could result in increased metabolic rate which could largely affect mitochondrial metabolism and potentially decrease efficiency (Rolfe et al., 1999). Multiple studies have demonstrated that.

caloric restriction decreases skeletal muscle proton leak (Bevilacqua et al., 2004; Bevilacqua et al., 2005). This is of note because our study demonstrated contrary findings since C2C12 myoblasts cultured in serum from RES offspring had increased proton leak. However, this result may be different if proton leak was measured in the mitochondria or in satellite cells isolated from RES offspring. Others have shown that poor maternal nutrition during gestation can alter metabolism in other stem cells (Pillai et al., 2016). Specifically, Pillai et al., (2016) reported that restricted feeding decreased basal respiration, ATP production, and maximal respiration of offspring mesenchymal stem cells compared with controls. Additionally, maternal over-feeding decreased basal respiration, ATP production, maximal respiration, and spare-respiratory capacity of offspring mesenchymal stem cells compared with controls. Although the variables altered were different than in the present experiment, the data demonstrate that poor maternal nutrition can alter stem cell metabolism in different cell populations. In mice, male offspring from obese mothers had decreased mitochondrial complex II-III (Shelley et al., 2009). Another group showed that pigs exposed in utero to high energy diets had decreased mitochondrial DNA content and downregulated mRNA expression of genes associated with mitochondrial biogenesis and function (Zou et al., 2016a). Further, moderate maternal dietary energy intake decreased mitochondrial biogenesis (Zou et al., 2016b). Despite noted differences in mitochondrial respiration in C2C12 myoblasts exposed to offspring serum, there were no changes to glycolytic function. Similarly, poor maternal nutrition during gestation did not alter any of the variables associated with glycolytic function in mesenchymal stem cells (Pillai et al., 2016). Therefore poor maternal nutrition may be altering metabolism through changes to mitochondrial function.

Metabolome Analysis

To better understand metabolic changes in muscle during gestation in response to poor maternal nutrition, a metabolome analysis was conducted on LM samples from offspring of control-fed, restricted-fed, and over-fed dams at d 90, d 135, and birth time points. Metabolome analysis demonstrated that both maternal diet and day of gestation drive changes to LM metabolism, including changes to amino acid, lipid, carbohydrate, and energy metabolism. The principle component analysis indicates that samples within each developmental time period (d 90, d 135, birth) exhibit close overlapping grouping, indicative of an overall similar metabolite composition. This suggests that the stage of development is a more powerful driver of metabolism than maternal nutrition. This is not unexpected given the vast changes in fetal muscle metabolism during gestation. Birth represents a switch in nutrient utilization and metabolism (Girard, 1990; Hillman, et al., 2012). The fetal diet is rich in carbohydrates supplied from the mother and poor in fat. In the fetus, gluconeogenesis and ketogenesis are absent or very low. At birth, the maternal glucose supply is removed and the diet becomes more reliant on lipids and less reliant on carbohydrates (Girard, 1990). Because of this, there are drastic changes to energy metabolism, with gluconeogenesis and ketogenesis becoming more active within 24 h of birth (Girard, 1990; Hillman et al., 2012). In our study, metabolome analysis demonstrated a similar shift in nutrient utilization between the fetus and postnatal animal.

Maternal over-nutrition during gestation alters offspring LM metabolome at d 90

At d 90 of gestation, offspring from over-fed mothers exhibited altered amino acid metabolism compared with controls demonstrated by changes to alanine and aspartate metabolism, tyrosine metabolism, tryptophan metabolism, histidine metabolism, and leucine, isoleucine and valine metabolism. Amino acid sensing can regulate myogenesis through the

mammalian target of rapamycin (mTOR) pathway (Mee-Sup and Chen, 2013). The mTOR pathway is a key regulator of skeletal myogenesis and regulates different steps of differentiation (Ge and Chen, 2012). Multiple studies indicate that amino acids work upstream in the mTOR signaling pathway and can serve to activate signaling through the pathway, resulting in increased protein synthesis (Sancak et al., 2008, Ma and Blenis, 2009, Sancak et al., 2010; Bar-Peled et al., 2012, Bar-Peled et al., 2013). Therefore, these early changes to amino acid metabolism, especially leucine metabolism, may play a part in regulating myogenesis.

Offspring of over-fed dams also exhibited changes to glutathione metabolism, including increased reduced glutathione (GSH) and decreased cysteine-glutathione disulfide and 2-aminobutyrate. However, the oxidized glutathione (GSSG) was similar to control. Changes to glutathione metabolism could be indicative of changes in redox status and oxidative stress. The GSH:GSSG ratio can be used as a marker of oxidative stress, and changes to this ratio are indicative of changes in redox status (Zitka et al., 2012). Therefore, follow up analysis of markers of oxidative stress and/or antioxidants in the muscle would further define the redox status in the offspring and the response to poor maternal nutrition.

Maternal over-feeding also resulted in perturbations to branched-chain amino acid (BCAA) catabolism. Offspring from over-fed ewes exhibited divergent branched-chain amino acid catabolism compared with controls. Specifically, LM from offspring of over-fed mothers exhibited accumulation of three key intermediates of BCAA breakdown, beta-hydroxyisovalerate, 3-methylglutaconate, and 3-hydroxyisobutyrate at both d 90 and 135 that indicate increased BCAA catabolism. This may reflect increased support for muscle energy production since the complete oxidation of the BCAAs in the mitochondria efficiently allows formation of ATP by oxidative phosphorylation (Brosnan and Brosnan, 2006). Less likely,

gluconeogenesis can also be supported, as the end-products of the pathway (succinyl CoA and acetyl CoA) can support both of these processes (Shimomura et al., 2004; Brosnan and Brosnan, 2006; Tao et al., 2017).

Maternal over-nutrition during gestation alters offspring LM metabolome at d 135

Similar to d 90, the most abundant metabolite changes at d 135 were to amino acid metabolism, including alanine and aspartate metabolism, N-acetylaspartate (NAA) metabolism, branched chain amino acid metabolism, and histidine metabolism. N-acetylaspartate is synthesized from aspartate and acetyl-CoA by aspartate N-acetyltransferase (Bogner-Strauss, 2017). It can then be cleaved by aspartoacylase to generate aspartate and acetate. Acetyl-CoA synthetase uses acetate to generate acetyl-CoA, a general energy metabolite and second messenger with roles in lipid synthesis (Bogner-Strauss, 2017). For histidine, imidazole propionate and formiminoglutamate, products of histidine catabolism, were increased compared with controls. There were also increases in metabolites involved in leucine, isoleucine and valine metabolism at d 135 compared with controls. Leucine, isoleucine, and valine are branched chain amino acids and are essential to muscle metabolism since they can be used as energy sources and are necessary for protein synthesis in the muscle (Wagenmakers et al., 1998). Branched chain amino acids stimulate protein synthesis and are required for mTOR regulation of myogenesis (Yoon et al., 2017). This pathway is essential for muscle hypertrophy and maintaining muscle mass (Yoon et al., 2017). At d 135, the muscle is no longer going through the primary and secondary waves of myogenesis, but instead starting to hypertrophy. Therefore, alterations to amino acid metabolism could negatively affect protein synthesis and skeletal muscle hypertrophy (Du et al., 2010).

Maternal over-nutrition during gestation alters offspring LM metabolome at birth

Over-feeding during gestation altered the lipid metabolism in the offspring at birth. Specifically, increased maternal nutrient consumption induces apparent lipid oxidation in the lambs. There was an increase in the ketogenic marker 3-hydroxybutyrylcarnitine at birth in offspring from over-fed mothers. This is associated with a decrease in short and long chain acylcarnitines. Carnitines shuttle long-chain fatty acids into the mitochondria where they are used to generate ATP (McGarry et al., 1975; Parvin and Pande 1978). They serve as substrates in branched chain amino acid metabolism and provide readouts for β-oxidation (McGarry et al., 1975; Parvin and Pande 1978). In general, ketogenesis increases when the rate of liver fatty acid β-oxidation exceeds the TCA cycles capacity to use acetyl-CoA. Ketogenesis frequently occurs under conditions of nutrient deprivation and high circulating free fatty acids (McGarry et al., 1975; Parvin and Pande 1978). Thus, changes to acylcarnitines in the LM of OVER offspring may reflect a mechanism to utilize excess β-oxidation intermediates. Corresponding depletion of glycolytic intermediates dihydroxyacetone phosphate (DHAP) and phosphoenolpyruvate (PEP) indicate reduced carbohydrate oxidation in favor of lipid utilization. During lipid synthesis, DHAP provides adipose cells with the activated glycerol backbone required to synthesize new triglyceride. Taken together with the depletion of polyunsaturated fatty acid concentrations of docosapentaenoate (n3 DPA; 22:5n3) and eicosapentaenoate (EPA; 20:5n3), this suggests a shift away from lipogenesis to increased lipid oxidation in offspring from over-fed mothers, relative to controls.

The lipid oxidation profile in offspring from over-fed mothers is also associated with cellular membrane degradation. Decreased concentrations of muscle phosphatidylcholines (PC)

and phosphatidylethanolamines (PE), including 1-linoleoyl-2-linolenoyl-GPC (18:2/18:3)* and 1-oleoyl-2-linoleoyl-GPE (18:1/18:2)* were identified compared with control offspring at birth. These lipids are key components of the plasma membrane and their depletion may represent increased membrane turnover (Van der Veen et al., 2017). A similar profile is produced in offspring of restricted-fed mothers that largely involves a distinct subset of PC, including 1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)*. Because these lipids are comprised of similar FA speciation, these decreases are likely associated with membrane degradation, relative to control animals, as both groups respond to changes in maternal diet.

Over-feeding during gestation also resulted in changes to the offspring lysolipid pool. Lambs from over-fed mothers exhibited depletion in the lysolipid pool at birth, compared with control offspring. The majority of the altered phospholipids are ethanolamine and choline derivatives (e.g. 1-palmitoleoyl-GPE (16:1)*, 1-palmitoyl-GPE (16:0), 1-linoleoyl-GPC (18:2), and 1-arachidonoyl-GPE (20:4n6)*) that are enriched on the inner and outer leaflets of the plasma membrane, respectively. Lysolipids, as well as fatty acids, are the natural products formed by the hydrolysis of phospholipids and may represent membrane remodeling related to phospholipid lysis (Arouri and Mouritsen, 2013). Collectively, offspring of over-fed dams exhibit potently increased β-oxidation, particularly at birth. This is of note since increased β-oxidation in muscle cells is associated with enhanced insulin-stimulated glucose metabolism and also protects against fatty acid-induced insulin resistance (Perdormo et al., 2004). The increased β-oxidation could be a potential response to increased maternal nutrition.

Maternal restricted-feeding during gestation alters offspring LM metabolome at d 90

At d 90, one of the most apparent changes in response to maternal restricted-nutrition was alterations to sphingolipid metabolism. Seventeen metabolites involved in sphingolipid metabolism were reduced compared with CON. Sphingolipids are an essential part of cellular lipid membranes and also serve as second messengers for signal transduction in pathways affecting cell growth, differentiation, stress responses, and death (Merrill et al., 1997; Khavander and Murshed, 2015). Sphingolipids play a role in the regulation of skeletal muscle development and tissue homeostasis (Stoffel, 1999; Aubin et al., 2005; Khavander et al., 2011, 2013; Khavander and Murshed, 2015). The broad changes to sphingolipid metabolism demonstrate the early effects of maternal restricted-nutrition on muscle development.

Additionally, there are changes to glutathione metabolism at d 90 in the offspring from restricted-fed mothers. Similar to OVER offspring at this time-point, GSH is increased 3.1-fold and 2-aminobutyrate is decreased, again suggesting changes in redox status. This could have potential negative effects on muscle metabolism. For example, oxidative stress has been shown to reduce insulin-stimulated glucose transport and insulin signaling in insulin-sensitive cells such as L6 myocytes (Maddux et al., 2001; Rudich et al., 1997; Rudich et al., 1998). Thus, there is potential for altered redox status to result in impaired insulin action and altered metabolism within the muscle.

Maternal restricted-feeding during gestation alters offspring LM metabolome at d 135

In RES offspring at d 135, amino acid metabolism, including histidine, branched chain amino acids, glutamate, tyrosine and tryptophan were altered compared with CON. Of particular interest is the reduction of glutamate in RES offspring compared with CON. Glutamate is essential for protein synthesis in the body (Walker and Van der Donk, 2016), but also has an

important role in muscle metabolism (Rennie et al., 1995). Muscle glutamate concentrations are correlated with muscle glutathione concentrations, and glutamate plays a determining role in glutathione synthesis (Amores-Sanches and Medina 1999; Engelen et al., 2000; Rutten et al., 2005). The reduction in glutamate concentrations at d 135 is of note since it corresponds with changes to glutathione metabolism at this time point. Specifically, GSSG is reduced and 2-aminobutyrate is increased in RES compared with CON. The reduction in glutamate may be one of the mechanisms promoting the change to glutathione metabolism, and therefore redox status, at this time point.

Maternal restricted-feeding during gestation alters offspring LM metabolome at birth

Restricted maternal feeding additionally altered offspring pyrimidine metabolism at birth. Pyrimidine intermediates provide important substrates for a wide array of lipids required for membrane synthesis and may provide insight into the apparently distinct lipid oxidation profiles (Berlin and Oliver, 1975; Lane and Fan, 2015). Here, there was an observed reduction in a number of pyrimidine derivatives, including uridine, cytidine, and cytidine diphosphate (CDP). Corresponding accumulation of pyrimidine biosynthesis marker orotate, with associated depletion of pyrimidine degradation markers β-alanine and 3-aminoisobutyrate, suggests increased pyrimidine synthesis. At this time point, there are also decreases to PC and PE pools. This could indicate decreased membrane synthesis, particularly in muscle tissue from offspring of mothers fed a restricted diet. Taken together with the changes to pyrimidine synthesis, this indicates that restricted-feeding may promote attenuated membrane and lipid synthesis, instead perhaps favoring anabolic reactions that favor pyrimidine synthesis.

Offspring from restricted-fed mothers also exhibit increased concentrations of the ketogenic marker 3-hydroxybutyrylcarnitine without associated significant changes in other lipids. Ketone bodies play an important role in the adaptation to fasting, serving as an important fuel for energy (Puckalska and Crawford, 2017). This increase in 3-hydroxybutyrylcarnitine at birth could point to changes in energy utilization within the muscle and a potential for ketosis postnatally. Offspring from restricted-fed mothers also exhibited changes to PC and PE metabolism, and decreased concentrations of lysolipids, providing some evidence for membrane turnover in these offspring. Again this demonstrates that RES offspring show changes to membrane lipids and lipid oxidation at birth.

Maternal nutrient restriction also promotes changes in offspring cholate metabolism. Prenatal caloric restriction results in reduced plasma bile acids in offspring mice, correlating with low birth weight, glucose intolerance, and obesity (Ma et al., 2017). In the current study, we observe divergent changes in cholate and a number of cholate bile salts, including taurocholate, and deoxycholate at d 90 in muscle tissue from both RES and OVER offspring. With no obvious changes in cholesterol metabolism, these changes likely do not reflect alterations in bile salt synthesis, but rather dietary influences that may alter nutrient utilization by the intestine. Further, there is evidence of perturbed enterohepatic circulation in the restricted group given increases in primary bile salts (e.g., glycochenodeoxycholate, taurochenodeoxycholate, and glycocholate), but decreased concentrations of secondary bile salts, particularly taurodeoxycholate. Briefly, primary bile acids are conjugated with either taurine or glycine to decrease toxicity and increase solubility. In the intestine, a portion of the bile acids could become deconjugated or modified to form secondary bile acids which are reabsorbed by enterohepatic circulation. Decreased muscle concentrations of secondary bile salts may reflect impairment of this process in the restricted

state and possible suggest alterations in fetal or maternal liver function (Joyce et al., 2011). This can have a significant impact on body weight gain and growth responses, as shown in a recent murine model (Joyce et al., 2011).

Conclusion

Although the day of gestation had a greater effect on metabolic change than the experimental feeding groups, analysis of the LM metabolome indicated that poor maternal nutrition alters muscle metabolism. Muscle tissue from offspring of over-fed mothers exhibited evidence of increased lipid oxidation accompanied by increased membrane turnover. Fetuses of over- and restricted-fed mothers demonstrated altered amino acid utilization which may result in changes to protein synthesis affecting muscle hypertrophy. Additionally, over- and restricted-feeding also led to altered muscle concentrations of cholate during development. Offspring from restricted-fed mothers demonstrated increased utilization of short chain fatty acids and increased pyrimidine anabolism at birth. Restricted maternal nutrition may impair enterohepatic circulation of bile salts in the offspring, which can have a profound impact on glucose tolerance and development. However, further analysis of tissues such as the liver or serum from offspring may give a clearer picture of changes to bile salts. Finally, offspring from both over- and restricted-fed ewes demonstrated altered glutathione metabolism at multiple time points, which could indicate altered redox status, and possible oxidative stress.

Future Directions

Although the metabolome data gave a detailed picture of metabolic change in response to maternal diet and day of gestation, further analysis on muscle tissue will provide additional insight. Specifically, alterations in glutathione metabolism suggest the need for additional

measures of redox status and oxidative stress. Additionally, the broad changes to lipid metabolism in response to maternal diet, especially at birth, indicate that histological analysis of lipid content within the muscle tissue may be warranted. Another area of interest is mTOR signaling since this pathway is partially regulated by amino acid sensing. The metabolome analysis indicated broad changes to amino acid metabolism in both treatment groups. Therefore, there is potential for mTOR to be affected. Analysis of the mTOR pathway could determine if the changes to amino acids are actually influencing mTOR regulation of protein synthesis. Overall, further analysis of the muscle tissue would support the metabolome findings and provided a more detailed understanding of how poor maternal nutrition during gestation is affecting muscle development and metabolism.

APPENDIX 1

Staining Protocol for Myosin Heavy Chain

First Day:

1. Remove cells from incubator
2. Remove media
3. Fix wells with 4% paraformaldehyde for 15 minutes
4. Rinse 2x with PBS
5. Add blocking solution for 20 minutes
    - 0.2% Triton-X and 5% horse serum in PBS
6. Add primary antibody
    - MF20 at a dilution of 1:100
7. Let sit overnight in the dark (wrapped in tinfoil) in the 4C fridge

Next Day:

1. Rinse 2x with PBS
2. Add secondary antibody for 1 hour
    - 488 goat-anti-mouse antibody at a dilution of 1:250
    - Hoescht 1:1000 to visualize nuclei
    - Keep in dark
3. Rinse all wells with PBS 2x
4. Image same day

APPENDIX 2

Seahorse XF Cell Mito Stress Test Kit Protocol

* Plate cells 24 or 48 hours before assay at correct density depending on experimental protocol C2C12 cells were plated at a density of 15,000 cells/ well and allowed to proliferate for 48 hours

Day prior to Assay

1. Plate cells at previously determined optimized density in the Seahorse XF Cell Culture Microplate using the appropriate cell culture medium.
2. Hydrate a sensor cartridge in Seahorse XF Calibrant at 37^{o}C in a non-CO2 incubator overnight.

Day of the Assay

Prepare Assay Medium

1. Turn on Seahorse XF analyzer at least one hour before beginning experiment and allow the machine to warm up and stabilize.
2. Prepare assay medium by supplementing Seahorse XF Base Medium with1 mM pyruvate, 2 mM glutamine, and 10 mM glucose as a starting point. However, desired medium concentration can be varied depending on cell type or in vitro culture conditions.
3. For 100 mL media add 1 mL pyruvate, 29.28 mg L-glutamine, and 0.4 mL glucose
4. Warm assay medium to 37^{o}C.
5. Adjust pH to 7.4 with 0.1 N with NaOH.
6. Keep at 37^{o}C until ready to use.

Prepare Stock Compounds

Note: *Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound.*

1. Prepare compounds using constant volume method provided in manual
2. Remove foil pouch from Seahorse XF cell Mito Stress Test Kit box. Each pouch contains reagents sufficient for one Seahorse XF Cell Mito Stress Test in a 24 well Seahorse XF Cell Culture Microplate.
3. Open pouch and remove the three tubes containing oligomycin (blue cap), FCCP (yellow cap), and rotenone/ antimycin A (red cap). Place tubes in small tube rack.
4. Re-suspend contents of each tube with prepared assay medium in volumes described in Table 2 of provided booklet with a pipette. Gently pipette up and down (~10 times) to solubilize compounds.
5. Prepare 3 mL of each compound in assay medium. Seahorse recommends using 1 uM of oligomycin for most cells.

Load Sensor cartridge

1. Standard assay – no additional injection:
    - Load compounds into the appropriate ports of a hydrated sensor cartridge for each well (75 µL per port)
        - Port A: Oligomycin
        - Port B: FCCP
        - Port C: Rotenone/ antimycin A

Prepare Seahorse XF Culture Microplate for Assay

1. Remove cells from culture microplates from the 37ºC CO2 incubator and examine cells under the microscope to confirm confluence.
2. Remove assay medium from water bath.
3. Change the cell culture growth medium I the cell culture microplate to warmed assay medium using a multichannel pipette and place the cell culture microplate into a 37ºC non-CO2 incubator for 45 minutes to 1 hour prior to the assay.

Run the Seahorse XF Mito Stress Test

- Open Wave and create new procedure file. Follow the instructions below:
    1. Design new experiment in wave
        - Add oligomycin, FCCP, and Rotenone/ Anitmycin-A to injection ports
        - Add treatment groups and add to plate map
    2. Select the "Review and Run" tab, then click "Start Run".
    3. When prompted, place the loaded sensor cartridge with the calibrant plate into the instrument, then click "I'm ready". Calibration will take approximately 15-30 minutes. **Note:** *remove cartridge lid and verify correct plate orientation.*
    4. Following calibration and equilibration of the cell culture microplate, when prompted click "I'm ready". Load the cell culture microplate and click "I'm ready" to run the assay.

Data Analysis

- The Seahorse XF Mito Stress Test Report Generator automatically calculates the Seahorse XF Cell Mito Stress Test parameters from Wave data that has been exported to Excel. It can be used either with standard or modified stress test protocol and provides convenient, customizable one-page assay summary. The Report Generator can be installed either alongside Wave or directly from the Seahorse Bioscience website.
- Next day: Isolate DNA from each well to normalize data using the Macherey-Nagel NucleoSpin Tissue Kit (50 preps; Ref #740952.50) according to manufacturer instructions

APPENDIX 3

Seahorse XF Cell Glycolysis Stress Test Kit Protocol

*Plate cells 24 or 48 hours before assay at correct density depending on experimental protocol C2C12 cells were plated at a density of 15,000 cells/ well and allowed to proliferate for 48 hours

Day prior to Assay

1. Plate cells at previously determined optimized density in the Seahorse XF Cell Culture Microplate using the appropriate cell culture medium.
2. Hydrate a sensor cartridge in Seahorse XF Calibrant at 37^{o}C in a non-CO2 incubator overnight.

Day of the Assay

Prepare Assay Medium

1. Turn on Seahorse XF Analyzer at least one hour before experiment and let it warm up to stabilize.
2. Prepare assay medium by supplementing Seahorse XF Base Medium with 1 mM glutamine as a starting point.
3. For 100 mL media add 14.6 mg L-glutamine.
4. Warm assay medium to 37^{o}C.
5. Adjust pH to 7.4 with 0.1 N with NaOH.
6. Keep at 37^{o}C until ready to use.

Prepare Stock Compounds

Note: *Use compounds the same day they are reconstituted. Do not refreeze. Discard any remaining compound.*

1. Prepare compounds using constant volume method described in provided manual
2. The Seahorse XF Glycolysis Stress Test Kit Includes 6 foil pouches each containing oligomycin, 6 vials containing glucose, and 6 vials containing 2-DG. The kit reagents are sufficient for 6 XF Glycolysis Stress Test assay in a 24-well Seahorse XF Cell Culture Microplate.
3. Open foil pouch containing oligomycin (light blue cap) and remove 1 vial containing glucose (blue cap) and 1 vial containing 2-DG (green cap) from the kit box.
4. Re-suspend each component with prepared assay medium in volumes described in 3 with p1000 pipette. Gently pipette up and down (~10 times) to solubilize the compounds. Vortex the 2-DG to ensure that it goes into solution.

Load Sensor cartridge

1. Standard assay – no additional injection:
    - Load compounds into the appropriate ports of a hydrated sensor cartridge for each well (75 µL per port)
        - Port A: Glucose
        - Port B: Oligomycin
        - Port C: 2-DG

Prepare Seahorse XF Culture Microplate for Assay

1. Remove cells from culture microplates from the 37ºC CO2 incubator and examine cells under the microscope to confirm confluence.
2. Remove assay medium from water bath.
3. Change the cell culture growth medium I the cell culture microplate to warmed assay medium using a multichannel pipette and place the cell culture microplate into a 37ºC non-CO_2 incubator for 45 minutes to 1 hour prior to the assay.

Run the Seahorse XF Glycolysis Stress Test

- Open Wave and create new procedure file. Follow the instructions below:
    1. Design new experiment in wave
        - Add glucose, oligomycin, and 2-DG to injection ports
        - Add treatment groups and add to plate map
    2. Select the "Review and Run" tab, then click "Start Run".
    3. When prompted, place the loaded sensor cartridge with the calibrant plate into the instrument, then click "I'm ready". Calibration will take approximately 15-30 minutes. **Note:** *remove cartridge lid and verify correct plate orientation.*
    4. Following calibration and equilibration of the cell culture microplate, when prompted click "I'm ready". Load the cell culture microplate and click "I'm ready" to run the assay.

Data Analysis

- The Seahorse XF Glycolysis Stress Test Report Generator automatically calculates the Seahorse XF Cell Mito Stress Test parameters from Wave data that has been exported to Excel. It can be used either with standard or modified stress test protocol and provides convenient, customizable one-page assay summary. The Report Generator can be installed either alongside Wave or directly from the Seahorse Bioscience website.
- Next day: Isolate DNA from each well to normalize data using the Macherey-Nagel NucleoSpin Tissue Kit (50 preps; Ref #740952.50) according to manufacturer instructions

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